UNIVERSIDADE ESTADUAL DE CAMPINAS

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Modulação dos Mecanismos de Defesa das Ilhotas Pancreáticas Contra o Estresse Oxidativo

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Índice

Resumo

No *diabetes mellitus*, radicais de oxigênio estão associados com perda de sensibilidade à glicose ou destruição das células β. Nesse trabalho, investigamos a tolerância de ilhotas de Langerhans de ratos neonatos ao estresse provocado por H_2O_2, seus mecanismos antioxidantes de defesa e os fatores que promovem manutenção da sensibilidade à glicose. Cultivadas com 1 mM de H_2O_2, as ilhotas aumentaram até 6× seu consumo de glicose e resistiram ao estresse induzido por H_2O_2 quando em meio contendo 20 mM de glicose. A expressão da enzima catalase em resposta à glicose se mostrou necessária a essa resistência, mas não suficiente. Em concentrações baixas de H_2O_2, quando a catalase tem sua atividade modulada por NADPH, observamos que mesmo proteínas sem atividade catalítica adquiriam capacidade antioxidante e eram regeneradas por NADPH. Mapeando a distribuição de atividade peroxidase na ilhota, observamos sensibilidade ao NADPH nas frações nuclear e citossólica.

As ilhotas cultivadas em 20 mM de glicose e as que resistem ao H_2O_2 possuem em comum uma maior atividade da via das pentoses, que gera NADPH citossólico. Nessas ilhotas, verificamos que a produção citossólica de NAD(P)H limita a secreção de insulina. Tais ilhotas produzem NAD(P)H principalmente da oxidação de substratos endógenos no citossol e nas mitocôndrias, ao invés de localizarem seu uso somente no citossol, como se dá nas ilhotas mais sensíveis ao H_2O_2. A cultura com 20 mM de glicose produziu ilhotas com alta expressão da lançadeira de NADH glicerol-fosfato, enquanto o H_2O_2 selecionou ilhotas com alta expressão da lançadeira mal/asp. Como ambas as lançadeiras promovem a comunicação entre citossol e mitocôndrias, concluímos que o sistema de lançadeiras e a geração de NADPH sejam fatores críticos para a manutenção da sensibilidade à glicose nas ilhotas.

Abstract

In *diabetes mellitus*, oxygen radicals are associated with loss of glucose-sensibility and destruction of β-cells. In this work, we investigated the tolerance of neonatal rat islets to stress induced by H_2O_2, the islets antioxidant defense mechanism and factors maintaining islet glucose-responsiveness. Islets cultured with 1 mM of H_2O_2 increased 6 fold the glucose uptake and resisted H_2O_2-induced stress when cultured in media containing 20 mM of glucose. Glucose-induced catalase expression was shown to be necessary to islet cell-survival, although not sufficient. In low H_2O_2 concentrations, the activity of catalase is dependent on NADPH and we observed that even proteins with no catalytic activity could be antioxidants regenerated by NADPH. Mapping the peroxidase activity in islets, we observed sensibility to NADPH in nuclear and cytossolic fractions.

Islets cultured with 20 mM of glucose and islets that survived after culture with H_2O_2 both showed increased activity of the pentose phosphate pathway, which generate cytossolic NADPH. Is theses islets, we verified that cytossolic production of NAD(P)H limits insulin secretion. Such islets generate NAD(P)H principally from oxidation of endogenous fuels in cytossol and mitochondria, in contrary of the most H_2O_2-sensible islets which use endogenous fuels exclusively in cytossol. Culture with 20 mM of glucose produced islets with high expression of the glycerol-phosphate NADH shuttle, whereas culture with H_2O_2 selected islets with high expression of the mal/asp shuttle. Since both shuttles promote interchange between cytossol and mitochondria, we have concluded that the shuttle system together with NAD(P)H generation ability are critical factors in maintaining islet glucose-responsiveness.

Abreviações

αKG	α-cetoglutarato
4AA	4 amino-antipiridina
CAT	catalase ($2H_2O_2 \Rightarrow 2H_2O + O_2$)
FCS	fetal calf serum (soro fetal bovino)
GOx	glucose oxidase (glicose + $O_2 \Rightarrow$ glicolato + H_2O_2)
GSIS	glucose-stimulated insulin secretion (secreção de insulina estimulada por glicose)
HRP	horseradish peroxidase (H_2O_2 + fenol + NADPH $\Rightarrow$ H_2O + quinona + NADP)
IGF-1,2	insulin-like growth factor 1 or 2
IL-1β	interleucina 1β
iNOS	inducible nitric oxide synthase (Arg + O_2 + 2NADPH $\Rightarrow$ NO + NADP + L-citrulina)
mG3PDH	glicerol-3-fosfato dehidrogenase mitocondrial (G3P + NADH $\Rightarrow$ diidroxi-acetona fosfato + NAD)
mGDH	glutamato dehidrogenase mitocondrial (glutamato + NADP $\Rightarrow$ αKG + NADPH)
mMDH	malato dehidrogenase mitocondrial (malato + NAD(P) $\Rightarrow$ oxalacetato + NAD(P)H)
MMI	membrana mitocondrial interna
Mn/CuZnSOD	isoformas da enzima superóxido dismutase (O_2^- + $2H^+ \Rightarrow H_2O_2$)
mPC	piruvato carboxilase mitocondrial (piruvato + CO_2 + ATP $\Rightarrow$ oxalacetato + ADP + P_i)
mPDH	piruvato dehidrogenase mitocondrial (piruvato + NAD + HS-CoA $\Rightarrow$ CO_2 + acyl-CoA + NADH)
NRR	NAD(P)H reduction rate
PARS	poly-ADP-ribosyl sinthase (agrupa resíduos de ADP-ribose em histonas)
PPP	pentose phosphate pathway (via das pentoses)
RSH, **RSSR**	tiol genérico, formas reduzida e oxidada
TCA	tri-carboxylic acid cycle (ciclo de Krebs)

Introdução

As células β pancreáticas têm sua atividade secretora de insulina fortemente vinculada ao metabolismo de glicose e outros substratos energéticos, sendo secreção de insulina aparentemente iniciada por ATP ou NAD(P)H produzidos na via glicolítica (1). O ATP produzido no ciclo de Krebs complementa o processo, acionando a maturação e transporte dos grânulos de insulina até a membrana.

Por outro lado, a célula β é carente de sistemas enzimáticos que degradem o montante de espécies reativas de oxigênio (EROs) gerado por situações patológicas, entre elas o *diabetes mellitus* tipo 1 (2). No *diabetes mellitus* tipo 2, a glicosilação não-enzimática de proteínas plasmáticas e seu reconhecimento por macrófagos alimenta um processo crônico de estresse oxidativo, que pode levar ao mal funcionamento das células β e perda da secreção de insulina estimulada por glicose (GSIS) (3).

A importância dos radicais de oxigênio na ilhota

A relação entre a atividade metabólica na célula B e sua menor proteção enzimática contra EROs talvez se deva ao fato de estes EROs agirem como reguladores da degradação de glicose. Ao interagirem com proteínas, esses EROs causam oxidação de resíduos de cisteína e de sítios ativos contendo metais de transição, assim inibindo enzimas-chave para a produção de ATP, como a fosfofrutoquinase e a aconitase (4).

Sabe-se que mesmo o acúmulo de certos metabólitos no citossol da célula β dá origem a EROs (5). A interação desses EROs com substâncias redutoras comumente origina OH^- e O_2^- (6). A célula β expressa MnSOD na matriz mitocondrial e CuZnSOD no citossol, que convertem tais radicais em H_2O_2. Na matriz, o H_2O_2 é degradado ou difunde-se para o citossol. A inibição do efluxo de H_2O_2 pela produção citoplasmática (7) ou o influxo de H_2O_2 exógeno facilitam a oxidação de tióis das proteínas nas membranas mitocondriais, provocando sua aglutinação como poros que comunicam a matriz mitocondrial, o espaço intermembranas e o citoplasma.(8). O mesmo H_2O_2 ativa a proteína desacopladora UCP2, prejudicando a formação de potencial na MMI (9). Gerando menos ATP, a célula passa a ter resposta diminuída a secretagogos como a glicose (10). Pelos poros abertos na membrana mitocondrial, ocorre entrada de líquido e vazamento para o citoplasma de citocromo C, iniciando a fragmentação do DNA nuclear por caspases (8). Muitos são os trabalhos que associam a inativação de EROs com proteção da GSIS, em ilhotas isoladas ou *in vivo* (11, 12, 13).

O H_2O_2 atravessa membranas lipídicas e as junções celulares na ilhota de Langerhans (14), podendo ser degradado na célula β, em outras células ou mesmo no plasma por proteínas como a catalase (CAT), tioredoxina (TSH), tioredoxina peroxidase (TPx) ou o sistema glutationa/glutationa peroxidase (GPx). A célula β apresenta MnSOD, TSH, TPx, glutationa (GSH) e GPx na matriz mitocondrial, além de tióis reativos na membrana mitocondrial externa, que participam da reação $2RSH + H_2O_2 \Rightarrow RSSR + 2H_2O$ (15). Até alguns anos atrás, cogitava-se que a catalase também pudesse fazer uso de NADPH em baixas concentrações de H_2O_2, uma vez que a molécula da enzima possui um sítio para a ligação deste (16). Nesse caso, as elevadas concentrações de NAD(P)H encontradas na célula β favoreceriam esse tipo de reação, além de propiciar a comunicação entre os diversos sistemas antioxidantes, que podem funcionar em sinergismo (17, 18).

O sistema varredor de EROs na ilhota

Mantendo concentrações de 12 a 25 μM no citoplasma e de 50 a 125 μM na matriz mitocondrial, a célula β produz grandes quantidades de NAD(P)H (19). O NADPH, gerado na via das pentoses, geralmente está associado à redução de tióis (20). Existem, no entanto, enzimas que utilizam NADH ou NAD(P)H e cujo equilíbrio cinético permite a conversão de uma forma em outra. Quando não consumidos no citossol, NADPH e NADH são transportados pelo envoltório mitocondrial por um sistema de lançadeiras, mantendo em equilíbrio as concentrações no citoplasma e na matriz. Além de facilitar o transporte de substâncias redutoras dentro da célula, sabe-se que tais lançadeiras são fundamentais ao metabolismo de glicose pela célula β (21).

O uso do NAD(P)H em reações ou sua menor formação inibem a síntese de ATP. Sabe-se que isso ocorre, na célula β, pelo consumo de NAD(P)H em reações com H_2O_2, por atividade da iNOS (nitric oxide sinthase) ou ainda por ativação da enzima nuclear PARS (poly-ADP-ribosyl sinthase). Enquanto a iNOS somente é expressa em resposta a processos apoptóticos, a PARS é ativada pela combinação de NO e DNA fragmentado (22, 23). Como resultado, há redução na secreção e síntese de insulina.

Os tióis pequenos como a glutationa ou mesmo a tioredoxina reagem com o H_2O_2, além de reduzir enzimas como as citadas, que possuem menor K_m. Teoricamente, tióis como L-cisteína, 2-mercaptoetanol e ditiothreitiol (DTT) que fossem internalizados pela célula poderiam reduzir o sítio ativo de tais enzimas. No entanto, são muito próximas as doses de tióis que são tóxicas ou protegem contra o H_2O_2 (24). Um aminoácido sulfonado menos reativo, a L-taurina aciona a maquinaria de redução de tióis na célula β, oferecendo proteção contra o H_2O_2 e derivados seus como o ácido hipoclórico (25, 26).

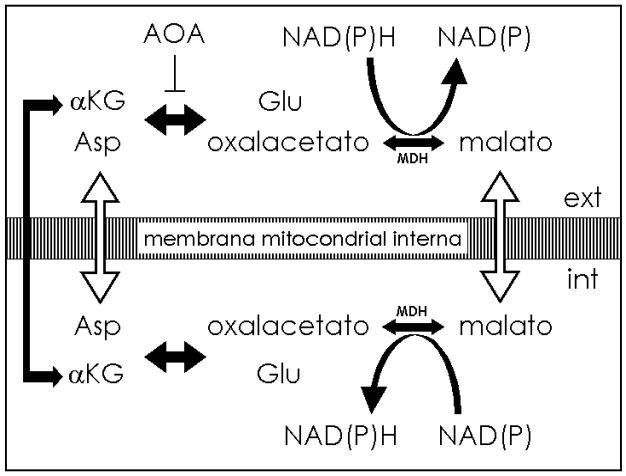
Além da catalase, que utiliza um sítio ativo contendo Fe^{3+}, outras enzimas ou mesmo proteínas associam-se a íons como Cu^{2+} e adquirem atividade peroxidase nas concentrações baixas de peróxido encontradas dentro da célula. Algumas dessas proteínas sofrem carbonilação e deaminação como resultado de reações com EROs, sendo inativadas e assim preservando enzimas-chave na célula β (27).

A função das lançadeiras de NADH na secreção de insulina

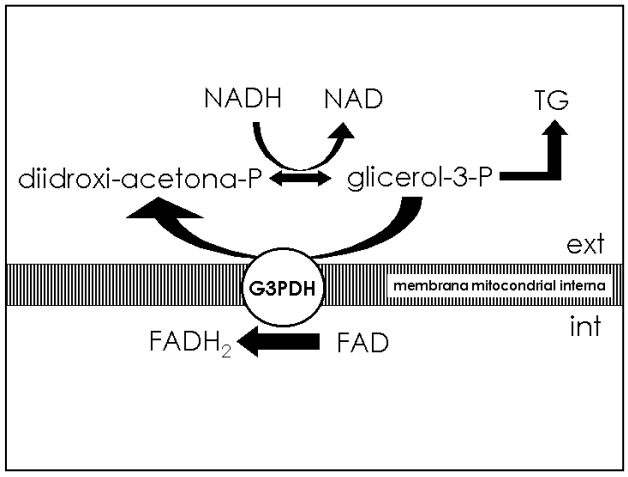
O NADH proveniente da glicólise age como um iniciador necessário da secreção de insulina (28), sendo transportado para a matriz mitocondrial através das lançadeiras piruvato/malato (pir/mal), citrato/isocitrato, malato/aspastato (mal/asp) e glicerol-fosfato. Enquanto a lançadeira pir/mal faz a conversão NADPH/NADH, a principal enzima da lançadeira glicerol-fosfato doa elétrons diretamente ao complexo II da cadeia respiratória ($FADH_2$-linked glycerol-3-phosphate dehydrogenase) (29).

Acredita-se que o NAD(P)H introduzido nas mitocôndrias dessa forma seja um iniciador de maior atividade no ciclo de Krebs, sem o qual não há polarização suficiente da membrana mitocondrial interna (MMI) para a geração de ATP (21). Estima-se que falta das lançadeiras diminua em até 50% a atividade no ciclo TCA. Em ilhotas de rato, esse mecanismo provém principalmente da lançadeira pir/mal, que introduz o piruvato derivado da glicólise na forma de oxalacetato, através da enzima piruvato carboxilase (mPC). Esse oxalacetato é convertido em malato pela enzima malato dehydrogenase (mMDH), sendo o malato largamente exportado para o citossol pelas mitocôndrias (30), o que o faz também um marcador da atividade mitocondrial (31).

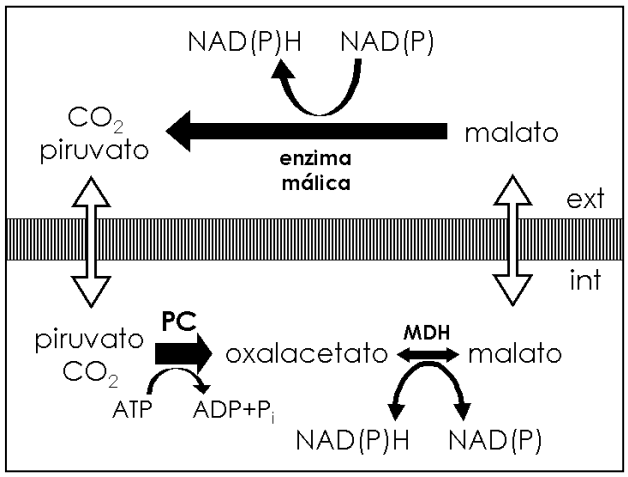
Nas ilhotas de rato, o malato é convertido em piruvato pela enzima málica, que mantém o citossol da célula β rico em piruvato e faz a conversão NADH/NADPH. Embora funcione como um mecanismo de perda do NAD(P)H mitocondrial, essa lançadeira está ligada à síntese de lípides e mostra-se essencial à secreção de insulina induzida por glicose (32). Cogita-se pouca atividade para a lançadeira citrato/isocitrato, uma vez que nenhum secretagogo induz exporte de isocitrato pelas mitocôndrias de células β (31).



A lançadeira malato-aspatato, acionada pela diferença de concentração de NAD(P)H entre o espaço intermembranas a matriz mitocondrial.



A lançadeira glicerol fosfato, que está relacionada à síntese de tri-glicerídeos e insere elétron do NADH diretamente no sítio 2 da fosforilação oxidativa.



A lançadeira piruvato/malato, relacionada ao transporte de NAD(P)H da matriz mitocondrial para o citoplasma.

A conexão entre a atividade mitocondrial e o sistema varredor de EROs

Embora existam poucos trabalhos sobre a mitocôndria da célula β, sabe-se que existe intenso tráfego de substâncias entre o citossol e a matriz mitocondrial (anaplerose) (31). Muitas dessas substâncias estão ligadas às lançadeiras de NADH, enquanto outras ao metabolismo de aminoácidos, que parece tamponar a atividade no ciclo de Krebs através da concentração de αKG, que é facilmente convertido em L-glutamato pela enzima glutamato dehidrogenase (mGDH) (33). Essa enzima é bastante expressa em células β, onde é ativada por L-leucina e inibida (fisiologicamente) pela relação GTP/ADP. Quando a célula β é estimulada por 16.7 mM de glicose, a mGDH aumenta em até 3× a concentração de glutationa reduzida. O metabolismo de L-glutamato ainda sugere que parte significativa dos carbonos derivados da glicose seja direcionada para a síntese de γ-glutamyl-cisteína, um precursor da glutationa (34).

O NADPH alimenta a redução de tióis como glutationa e tiorredoxina, de modo que a disponibilidade de NADPH limita a atividade do sistema antioxidante na célula β. Esse sistema reverte a oxidação das proteínas celulares por H_2O_2 e seus derivados, de modo que um aumento de H_2O_2 na célula ou a insuficiência do sistema antioxidante tem efeito deletério sobre as células da ilhota (8, 10). Dentre as ilhotas isoladas e cultivadas com H_2O_2, no entanto, observa-se que um certo número tolera e adapta-se ao H_2O_2 sem perda da GSIS. Esse trabalho buscou revelar os motivos dessa resistência e os mecanismos pelos quais as ilhotas protegem-se contra o agente deletério, como forma de orientar futuros trabalhos para a prevenção do *diabetes mellitus*.

Objetivo central do trabalho

Relacionar o metabolismo das células β com sua resistência ao H_2O_2.

Trabalho nº 1

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Protective effect of D-glucose, L-leucine and fetal calf serum against oxidative stress in neonatal pancreatic islets

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Abstract

B-cell destruction during the onset of diabetes mellitus is associated with oxidative stress. In this work, we investigated the mechanisms of defense against oxidative stress present in neonatal islets and their modulation by D-glucose, L-leucine and fetal calf serum (FCS). Culturing neonatal rat islets in the presence of low D-glucose concentrations (2.8–5.6 mmol/l) and 1 mmol/l H_2O_2 increased the D-glucose uptake by islets sixfold compared to control levels. This effect was dose-dependently inhibited by D-glucose or FCS and by high concentrations of L-leucine. These supplements allowed islets to increase cytoplasmic catalase (CAT) activity only in response to H_2O_2, with no decrease in NO formation. Although L-leucine increased CAT activity and restored D-glucose uptake, it did not prevent damage to the islets. These data indicate that the most important H_2O_2 scavenger system in the islets is CAT and that this system can be modulated by metabolic substrates.

Keywords: Neonatal pancreatic islet; D-Glucose; L-Leucine; Fetal calf serum; Oxidative stress; H_2O_2; Catalase

1. Introduction

Pancreatic B-cells have low expression of reactive oxygen species (ROS) scavenger enzymes such as superoxide dismutase (SOD), catalase (CAT) and gluthathione peroxidase (GPx) [1], and oxidative stress in these cells is common [2]. The onset of type 1 diabetes is partly mediated by H_2O_2 released from activated lymphocytes that infiltrate islets [3–5]. In contrast, type 2 diabetes evolves to advanced clinical complications through islet damage and loss of insulin secretion, mediated by gluco-lipo-toxicity, TNFα cytotoxicity, and recognition of advanced glycated ends (AGEs) by islet resident macrophages and their subsequent activation [6–8].

Oxidative stress in B-cells occurs through different pathways [9]. Since B-cells express CuZnSOD (cytoplasm) and MnSOD (mitochondrial matrix) [10], they decompose ROS into H_2O_2 [11] but are unable to remove H_2O_2 because they lack a powerful peroxidase activity. Thus, H_2O_2 may accumulate in the cytoplasm and inhibit its own diffusion from the mitochondrial matrix [12]. Within the matrix, H_2O_2 causes lipid peroxidation [9] and provokes the cross-linking of thiol residues of inner mitochondrial membrane (IMM) and matrix proteins. Following oxidation, the ADP/ATP translocator, the major component of IMM proteins, interacts with proteins such as the voltage-dependent anion channel (VDAC) of the external mitochondrial membrane (EMM), cyclophilin D from the matrix, and ER proteins of the Bax and Bcl-2 families [12]. They form membrane permeability transition pores (MPTP) responsible for the decrease in IMM voltage, mitochondrial swelling and respiratory inefficiency [13].

In addition, cytoplasmic indicators of oxidative stress such as NIK and MEKK1 activate the transcription of iNOS and apoptotic genes [16–18]. iNOS uses NADPH, O_2 and L-arginine to increase NO production several fold [16,19], which in turn down-regulates the glycolytic and citric acid pathways [20,21], decreases cytochrome *c* oxidase complex

Abbreviations: SOD, superoxide dismutase; CAT, catalase; GPx, gluthathione peroxidase; TPx, thioredoxin peroxidase; BSA, bovine serum albumin; EDTA, ethylenediamine tetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; DTT, dithiothreithiol

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(COX) free-eletron scavenger activity [22,23] and alters nuclear signaling by increasing DNA exposure [24,25].

Enhanced generation of H_2O_2 and ROS in islets stimulates the transcription of apoptotic genes that results in mitochondrial and nuclear DNA fragmentation, inhibition of protein synthesis and reduction in islet cell mass. All of these processes can be prevented by fetal calf serum (FCS) or its components such as IGF-1, IGF-2 and GH [26,27].

In this work, we analyzed the effect of chronic exposure to D-glucose, L-leucine and FCS on the H_2O_2 scavenging system of neonatal rat islets. Our results indicate that all three components increase the rate of H_2O_2 degradation when H_2O_2 is present in culture media. Almost all the peroxidase activity detected was due to CAT. None of these substances reduced NO formation by islets exposed to H_2O_2. Finally, D-glucose and FCS, but not L-leucine, protected the islets against exposure to H_2O_2.

2. Material and methods

2.1. Islets isolation and culture

Neonatal (1–2 days old) and adult (4–6 months old) Wistar rats were purchased from the State University of Campinas animal facilities. After decapitation, neonatal [28] and adult [29] islets were isolated by collagenase (EC 3.4.24.3) digestion of pancreata in Hanks balanced salt solution. Islets were handpicked under a microscope, washed twice in sterile Hanks solution and cultured in RPMI 1640 medium (Sigma) supplemented with 2 g $NaHCO_3$/l and 1% penicillin/streptomycin, pH 7.4, and other supplements as indicated in the legends. Approximately 1000 islets/dish were maintained at 37 °C in a humidified atmosphere with 3% CO_2 for different periods of time. The medium was renewed every 48 h, as required.

2.2. NO production

NO formation was quantified by measuring the nitrite content of islet extracts. The islets in each dish were transferred to Eppendorf tubes containing 100 μl of Hanks solution (4 °C) and sonicated. The NO_2^- concentrations of total extracts were measured colorimetrically using a modified Griess reagent (1 part of 15 mg of sulfanilamide/ml in pure H_3PO_4 + 1 part of 1.5 mg NEED/ml in H_2O), using $NaNO_2$ dissolved in Hanks solution as standard. Absorbances were calculated as the difference between the readings at 540 and 600 nm. Assays were done immediately after recovery the islets from the dishes. NO_2^- levels were expressed as $[NO_2^-]$/[total protein] ratio.

2.3. D-Glucose uptake

D-Glucose concentrations in the media were determined using a commercial glucose enzymatic kit (Biotrol Diagnostic, Chennevières-lès-Louvres, France). D-Glucose uptake by the islets was measured as the variation in D-glucose concentrations in 10 ml of medium containing 5.6 mmol of D-glucose/l + 5% FCS at the end of 12 h in culture. Samples for D-glucose measurements were taken after filtration of the media through 0.45-μm pore filters (Millipore) to remove cells. The results were expressed as the D-glucose consumed/total protein content of islets in each dish. Extracts were prepared in Hanks solution by mechanical high-speed disruption of islets, at the end of the culture.

2.4. Peroxidase activity

Islet extracts were centrifuged at $10000g \times 6$ min to remove cellular membranes and organelles. The protein concentration of the liquid phase was measured (Biorad) and adjusted to 100 μg/ml by adding Hanks solution. H_2O_2 was added to each sample to a final concentration of 2.3 mmol/l and its concentration was then measured 0, 5, 10, 20, 30, 40 and 60 min later using a colorimetric reaction (Glucose Enzymatic Colour Liquide), against an H_2O_2 standard curve. The values were fitted to single exponential decay curves using the expression $[H_2O_2](t)=[H_2O_2]_{t=0} \times e^{-kt}$. The peroxidase activity of each sample was expressed as the k value (decay constant) obtained with given protein concentrations (100 μg/ml). Since the experiments with pure CAT showed a nonlinear crescent relationship between enzyme concentration and peroxidase activity, activities were not normalized.

2.5. Enzymatic kinetics

The velocity of H_2O_2 decomposition by islet protein or pure CAT (EC 1.11.1.6, H_2O_2 oxidoreductase) (Sigma) was determined in Eppendorf tubes containing either islet protein or CAT dissolved is Hanks solution alone with increasing concentrations of H_2O_2 (0 to 300 mmol/l). Aliquots of the medium were taken 1, 2, 3 and 4 min after H_2O_2 addition, diluted with acidified Hanks solution (pH 1.0) and assayed for H_2O_2, as stated. Initial H_2O_2 concentrations and the degradation rate were determined by linear regression over the data. K_m values were calculated from Boltzman sigmoidal curves fitted to the points.

2.6. Statistical analysis

The results were expressed as the means ± S.E. and the significance of differences was assessed by Student's t test. A value of $P<0.05$ indicated significance.

3. Results

Peroxidase activity in neonatal islets was approximately three times greater than adult islets ($k = 0.111 \pm 0.007$ min^{-1} vs. $k = 0.034 \pm 0.004$ min^{-1}, respectively, at 100 μg protein/

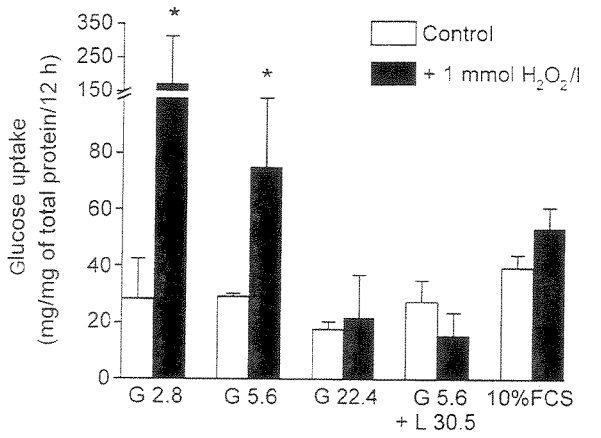

Fig. 1. Effect of H_2O_2 on D-glucose use by cultured neonatal islets. Islets were cultured for 4 days in RPMI medium with or without 1 mmol of H_2O_2/l and different concentrations of D-glucose (G; 2.8, 5.6 and 22.4 mmol/l) + 5% FCS, L-leucine (L; 30.5 mmol/l) + 5.6 mmol of D-glucose/l and 5% FCS or FCS (10%) + 5.6 mmol D-glucose/l. This medium was subsequently replaced by one containing 5.6 mmol of D-glucose/l and 5% FCS. The bars represent cumulative D-glucose use for 12 h as described in Section 2. The values are the means ± S.E. of six independent experiments. * $P<0.05$ vs. the respective control.

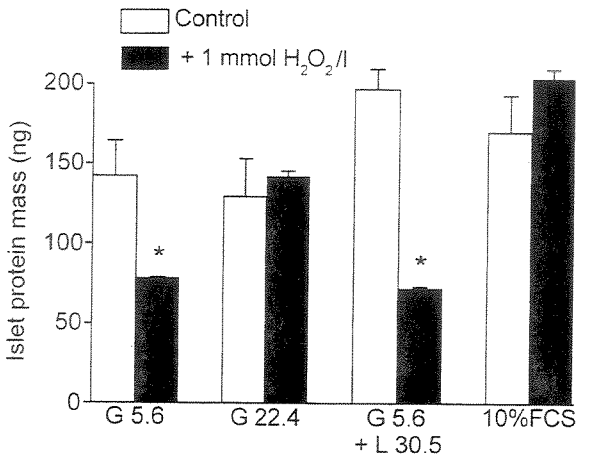

Fig. 3. Effect of H_2O_2 on protein mass of cultured neonatal islets. Islets were cultured for 4 days in RPMI medium with or without 1 mmol of H_2O_2/l and different concentrations of D-glucose (G; 5.6 and 22.4 mmol/l) + 5% FCS, L-leucine (L; 30.5 mmol/l) + 5.6 mmol of D-glucose and 5% FCS or FCS (10%) + 5.6 mmol of D-glucose/l. Subsequently, groups of 200 islets were sonicated in 100 μl of Hanks solution and the protein concentration was measured by a colorimetric assay. The bars represent the means ± S.E. of six independent experiments. * $P<0.05$ vs. the respective control.

ml, $n=4$; $P<0.05$). This activity was almost attributed to hydrophilic proteins since the removal of membranes and organelles from islet extracts did not alter the ability to decompose H_2O_2 ($k=0.24\pm0.02$ min^{-1} for the liquid phase vs. $k=0.22\pm0.02$ min^{-1} for the total extract, at 200 μg protein/ml, $n=4$; NS). Heating the extracts at 100 °C for 5 min reduced peroxidase activity to less than 3% of normal values, indicating that heat-denatured proteins are responsible for H_2O_2 degradation.

The presence of 1 mmol H_2O_2/l in culture medium containing low concentrations of D-glucose (2.8 mmol/l) dramatically increased the uptake of this sugar by neonatal islets (Fig. 1). This effect was significantly reduced by physiological concentrations of D-glucose (5.6 mmol/l)

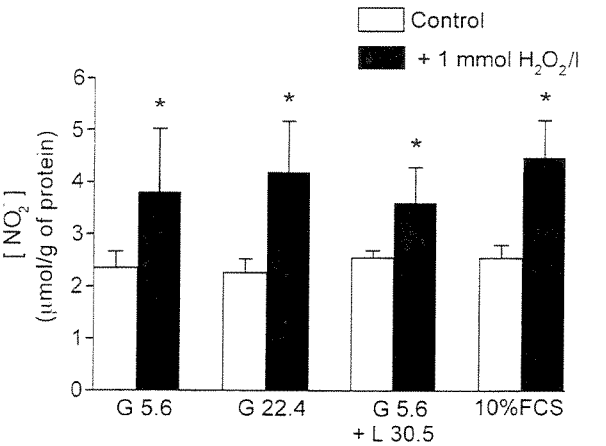

Fig. 2. Effect of H_2O_2 on nitrite production by cultured neonatal islets. Islets were cultured for 4 days in RPMI with or without 1 mmol of H_2O_2/l and different concentrations of D-glucose (G; 2.8, 5.6 and 22.4 mmol/l) + 5% FCS, L-leucine (L; 30.5 mmol/l) + 5.6 mmol of D-glucose/l and 5% FCS or FCS (10%) + 5.6 mmol of D-glucose/l. After culture, the islets were sonicated and NO_2^- concentrations within the cells were measured as described in Section 2. The values are the means ± S.E. of six independent experiments. * $P<0.05$ vs. the respective control.

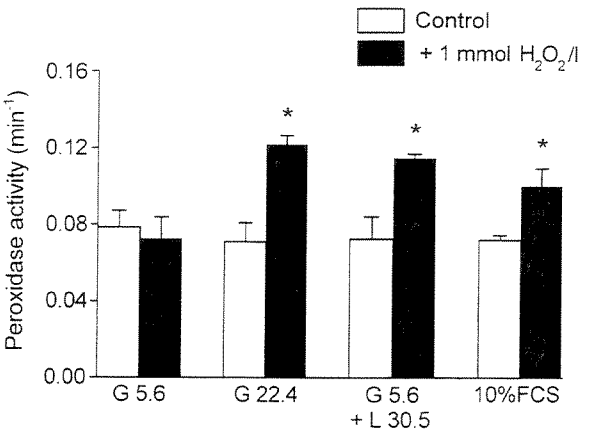

Fig. 4. Effect of H_2O_2 on peroxidase activity of cultured neonatal islets. Islets were cultured for 4 days in RPMI medium with or without 1 mmol H_2O_2/l and different concentrations of D-glucose (G; 2.8, 5.6 and 22.4 mmol/l) + 5% FCS, L-leucine (L; 30.5 mmol/l) + 5.6 mmol of D-glucose/l and 5% FCS or FCS (10%) + 5.6 mmol of D-glucose/l. The islets were subsequently sonicated and the peroxidase activity in the extract was measured as described in Section 2. The bars are the means ± S.E. of 10 independent experiments. * $P<0.05$ vs. the respective control.

Table 1
Effects of different concentrations of D-glucose and FCS on the peroxidase activity of islet extracts

Agents		Peroxidase activity (%)
D-Glucose (mmol/l)	FCS (%)	
2.8	5	116 ± 9
5.6	5	100
11.2	5	110 ± 30
22.4	5	70 ± 20 *
5.6	2	130 ± 6 *
5.6	5	100
5.6	7	85.4 ± 0.1 *
5.6	10	60 ± 20 *

The values are the means ± S.E. of four experiments.
* $P<0.05$ compared to the respective controls (5.6 mmol/l for D-glucose and 5% for FCS).

and abolished by 22.4 mmol of D-glucose/l, by the combination of D-glucose (5.6 mmol/l) plus L-leucine (30.5 mmol/l) and by 10% FCS (Fig. 1). H_2O_2 per se did not affect the D-glucose concentration in the incubation medium.

Exposure to 1 mmol of H_2O_2/l also increased nitrite production by the islets. This occurred even in the presence of D-glucose, L-leucine or FCS (Fig. 2). There was no correlation between D-glucose uptake and nitrite production, indicating that NO formation may not be responsible for increased D-glucose uptake by islets exposed to H_2O_2.

Culturing the islets in the presence of 1 mmol of H_2O_2/l and 5.6 mmol of D-glucose/l reduced the islet mass by 50% compared to the controls (absence of H_2O_2). High D-glucose (22.4 mmol/l) or FCS (10%), but not L-leucine (30.5 mmol/l), protected islet against damage by H_2O_2 (Fig. 3). Fig. 4 shows that the peroxidase activity of islet extracts was significantly increased by the exposure to H_2O_2, however, this increased activity was dependent on the presence of high D-glucose, L-leucine or FCS.

Unexpectedly, incubation of islets with high D-glucose and FCS concentrations in the absence of H_2O_2 dose-dependently reduced the peroxidase activity of islet extracts (Table 1).

In the next series of experiments, we investigated the enzyme responsible for H_2O_2 degradation in islet extracts. Fig. 5A shows that the enzyme activity of the extract did not change during successive challenges with H_2O_2, and that the incubation of samples with thiol reductants such as L-cysteine (1 mmol/l), 2-mercaptoethanol (1 mmol/l) or dithiothreithiol (DTT) (0.5 mmol/l) between two H_2O_2 additions did not affect the peroxidase activity of the extracts. Fig. 5B illustrates that addition of different inhib-

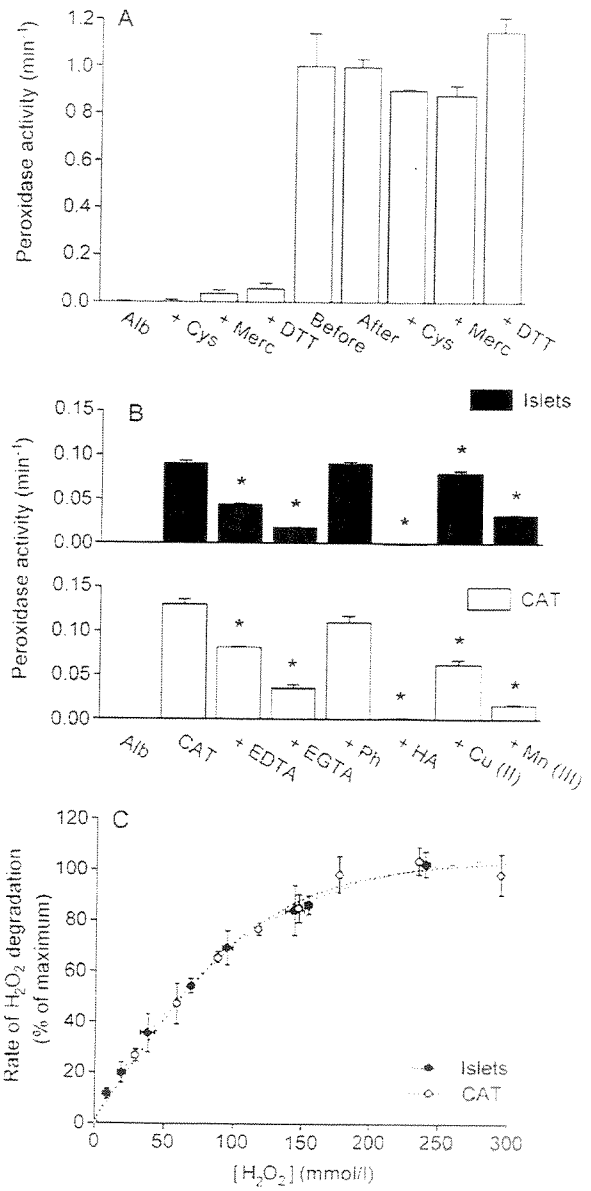

Fig. 5. Nature of the peroxidase activity in pancreatic neonatal islets. Islets were cultured in RPMI medium containing 5.6 mmol of D-glucose/l and 5% FCS. (A) represents the peroxidase activity of islet extracts (200 μg of protein/ml), the first challenge with H_2O_2 (before) and at the second challenge after a 30-min incubation with the following substances at the indicated final concentrations (pH adjusted to 7.4) in Hanks solution: none (After), 1 mmol of L-cysteine/l (Cys), 1 mmol of 2-mercaptoethanol/l (Merc) and 0.5 mmol of DTT/l (DTT). Negative controls were BSA (Alb: 100 μg/ml) without or with the same additions. The values are the means ± S.E. of six independent experiments. (B) (upper panel) represents the peroxidase activity of islet extracts (500 μg of protein/ml) in the presence of EDTA (40 mmol/l), EGTA (40 mmol/l), phenantroline (Ph) (10 mmol/l), hydroxylamine (HA) (10 mmol/l), and saturated $CuCO_3$ and $Mn_2(CO_3)_3$ (pH adjusted to 7.4). BSA (Alb) (100 μg/ml) was used as negative control. Identical experiments were done with purified catalase (lower panel). The values are the means ± S.E. of six independent experiments. * $P<0.05$ vs. control values (no additions). (C) represents the rate of H_2O_2 degradation measured at increasing concentrations of H_2O_2 using islet extracts or purified catalase (5.4 μg/ml). The K_m for the islet extracts and purified catalase were 70 ± 1 and 70 ± 0.8 mmol/l, respectively ($n=5$).

itors of CAT similarly reduced the peroxidase activity of islet extracts (upper panel) and of purified CAT (lower panel). Finally, the rate of H_2O_2 degradation for islet extracts and purified CAT had identical kinetics with K_m close to 70 mmol/l (Fig. 5C). The optimal pH for the islet extracts and purified CAT was 8.5.

4. Discussion

The best known ROS scavenging system in pancreatic islets consists of: COX, which avoids the leakage of free electrons from the respiratory chain [23]; SOD isoforms, which convert ROS into H_2O_2 in the cytoplasm or inside mitochondria; glutathione/glutathione reductase, which uses NAD(P)H as an electron source to regenerate glutathione within mitochondria; and CAT, which decomposes H_2O_2 into $H_2O + 1/2\ O_2$ [1]. We have also recently observed the presence of thioredoxin peroxidase (TPx) in islets that may represent another system for cytoplasmic H_2O_2 degradation (unpublished data).

As shown here, neonatal islets were protected better against oxidative stress than adult islets because of their more active peroxidase system. This greater protective ability against oxidative stress may be linked to higher circulating levels of growth factors in neonatal rats, thus allowing the islets to increase the rate of H_2O_2 degradation, if H_2O_2 is generated in large amounts (Fig. 4). Since this peroxidase system is almost totally localized in the water-soluble cell fraction, it is likely to be different from the peroxidase system of mitochondrial membranes [14].

Oxidative stress induced by H_2O_2 uncouples mitochondria by forming MPTP [14]. Thus, islets exposed chronically to H_2O_2 would be expected to metabolize higher amounts of D-glucose than controls in order to generate similar quantities of ATP, even at a nonstimulatory D-glucose concentration. In the presence of stimulatory concentrations, more ATP would be required for synthesis, maturation and translocation of insulin to the membrane [30]. The addition of D-glucose, L-leucine or FCS to the media containing H_2O_2 enhanced H_2O_2 degradation within the islets, thus decreasing the metabolic demand for ATP synthesis (Figs. 1 and 4).

NO has recently been associated with a reduction in COX activity that could increase ROS and H_2O_2 formation [23]. NO also reacts with ROS or H_2O_2 generated in mitochondria to form $ONOO^-$, an even more active radical [21]. Oxidative stress causes IkB heterodimer phosphorylation, subsequent NF-kB translocation to the nucleus and transcription of iNOS, in a positive feedback mechanism [17,31]. Since D-glucose, L-leucine and FCS did not decrease NO production in islets, with or without H_2O_2 in the culture media (Fig. 2), they probably failed to reduce iNOS expression. However, even with greater NO production, glucose uptake by islets was normalized in the presence of high concentrations of D-glucose, L-leucine and FCS (Fig. 1), indicating that increased NO production was not sufficient to induce mitochondria damage [21,32,33].

Thiol-reducing enzymes probably participate strong in H_2O_2 degradation by accelerating the reaction $2R\text{-}SH + H_2O_2 \Rightarrow RSSR + 2H_2O$ [15]. These enzymes and cofactors such as glutathione are reduced to the R-SH form by small thiol molecules such as L-cysteine, 2-mercaptoethanol and DTT [34]. However, treatment of samples with these thiol groups after exposure to H_2O_2 did not alter peroxidase activity, indicating that at the high concentrations of H_2O_2 used in our experiments [4], these enzymes and cofactors have a marginal participation in the degradation of H_2O_2 (Fig. 5A). To investigate the nature of peroxidase activity in islets, we used nonspecific inhibitors of metal-dependent enzymes such as ethylenediamine tetraacetic acid (EDTA), ethylenediamine tetraacetic acid (EGTA), Cu^{2+}, Mn^{3+}, phenantroline and hydroxylamine [35,36]. The variations in the peroxidase activity of islet extracts with each inhibitor mimicked the variations seen with purified CAT. These results, which were confirmed by the enzyme kinetics, strong indicated that this peroxidase was CAT (Fig. 5B,C).

Antioxidant systems must be regenerated by electron donors such as NAD(P)H, $FADH_2$, glutathione, DTT or other thiol groups from soluble (e.g. thioredoxin) or membrane-bound proteins (EMM) [15,34]. In normal cells, the concentration of H_2O_2 is in the range of 1 μmol/l, and CAT has to be coupled to NADPH to be active. In contrast, in the range of 1 mmol of H_2O_2/l, as used in our experiments and usually found in lymphocyte-infiltrated islets [3], CAT no longer requires NADPH [37]. NADPH levels, kept elevated by metabolic substrates (D-glucose and L-leucine), reduced the peroxidase activity in islets (Table 1). Thus, the decreasing amount of CAT seen in the presence of increasing concentrations of D-glucose or FCS may correlate with an increase in its individual molecules activity [37,38].

The increase in peroxidase activity produced by FCS suggests another possible mechanism in the regulation of scavenging systems. FCS is known to preserve islets by suppressing apoptotic genes and stimulating the transcription of anti-apoptotic ones [39]. This is the case of some Bcl-2 family proteins that inhibit the formation of MPTP [27]. Proteins with increased expression in response to growth factors may exert other protective effects against oxidative stress that cannot be explained simply by increasing CAT activity (Figs. 3 and 4). Our data indicate that, in the case of severe oxidative stress induced by H_2O_2 in islets, high concentrations of D-glucose, but not L-leucine (which shares the mitochondrial part of the metabolic pathway with D-glucose), may exert an FCS-like protection through some as yet unknown mechanism [38,40].

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Characterization of the peroxidase system at low H_2O_2 concentrations in isolated neonatal rat islets

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Abstract

B cell destruction during the onset of diabetes mellitus is associated with oxidative stress. In this work, we attempted to further trace the fate of H_2O_2 inside the pancreatic islets and determine whether it is mediated by enzymatic (peroxidase) activity or by chemical reaction with thiols from any protein chain. Our results suggest that the islet cells have a very similar peroxidase activity at the hydrophilic (cytoplasm) and hydrophobic compartments (organelles and nucleus), independent of the catalase content of the samples. This activity is composed of sacrificial thiols and by proteins with Fe^{3+}/Mn^{3+} ions at non-heme catalytic sites. The capacity of the hydrophobic fraction to scavenge O_2^- was increased in the presence of high concentrations of $NADP^+$ and RS^- and was highly dependent on RSH. On the contrary, the hydrophilic fraction exhibited a low RSH-dependent activity where the O_2^- scavenging is related to metal $Cu^{2+}/Fe^{3+}/Mn^{3+}$ ions attached to the protein molecules.

Keywords: NADPH; Neonatal pancreatic islet; Oxidative stress; Peroxidase; H_2O_2

1. Introduction

Pancreatic islets have a high glucose uptake [1], what accounts for a high production/detoxification of reactive oxygen species (ROS) [2]. Since ROS modulate the activity of important enzymes in glycolysis and TCA cycle, such as phosphofrutokinase and aconitase [3,4], an excess of either pro- or antioxidants may affect the regulation of islet cells metabolism. ROS are mostly generated by electron leakage from transport to molecular oxygen at the internal mitochondrial membranes, fat acid metabolism inside peroxisomes or specific membrane enzymes such as NADPH oxidase [5].

In the mitochondrial matrix, ROS are scavenged by MnSOD (which converts O_2^- into H_2O_2) or glutathione (GSH) and glutathione-binding enzymes that dimerizes and finally use NAD(P)H to recover the active form [6,7]. The H_2O_2 originated from other ROS is less active as an oxidant and can escape to the cytoplasm through the phospholipid membranes [8], where it is decomposed by catalase, glutathione/glutathione-linked, and thioredoxin/thioredoxin-linked enzymes. H_2O_2 may accumulate in the matrix provoking the peroxidation of lipids and thiol cross-linking of proteins in the inner mitochondrial matrix [9]. The H_2O_2 found inside the cells is predominantly generated through dismutation of O_2^- by CuZnSOD (in the cytosol), MnSOD (in the mitochondrial matrix) and/or comes from the outside, produced by activated lymphocytes [5]. When accumulated in the cytoplasm, H_2O_2 reduces the flow of H_2O_2 from the mitochondrial matrix and could be transformed back into O_2^- and OH$^\cdot$ through Fenton reactions with Fe^{2+} and Cu^+ ions [10]. Stressed B cells lose their efficiency in generating ATP from glucose and, as a consequence, the ability to secrete insulin. Finally, this situation activates apoptosis, leading to diabetes [6,11,12].

Compared with other islet cell types, the B cells are poor in scavenger enzymes, specially catalase [6], although the whole islet may increase its expression in response to oxidative stress, when cultured in the presence of certain metabolites [11]. On the other hand, insulin-secreting cells are known to produce high amounts of NAD(P)H from

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glucose metabolism, which varies from 12 to 25 μmol/l in the cytoplasm and from 50 to 125 μmol/l inside the mitochondria [13].

The H_2O_2 degradation through NAD(P)H-dependent enzymes is a source of NAD(P)$^{\bullet}$ radicals that may produce $O_2^{\bullet-}$ or inactive oxidized enzymes [14,15]. Since NAD(P)H sources are involved in the protection of cells when challenged by oxidative stress [16,17], we generated high reactive radicals such as RS$^{\bullet}$ (which binds to the narrowest thiol) and NADP$^{\bullet}$ (which is rapidly converted to $NADP^+ + O_2^{\bullet-}$) to simulate the fate of H_2O_2 products. These radicals were produced through the partial oxidation of NADPH or RSH at the conversion of HRP compound 1 (HRP[Fe^{4+}O][PH^+]) to compound 2 (HRP[Fe^{4+}O][PH]), which regenerates the rest of HRP by production of a colored quinone. The amount of radicals generated was chosen in the concentration range of NADPH and RSH found in B cells [18]. Also, we have analyzed the efficiency of the islet enzymatic system to decompose low concentrations of H_2O_2 (5.7 ± 0.7 μmol/l) and estimated the precise location of the key scavenger systems.

2. Materials and methods

2.1. Islets isolation and culture

When not specified, chemicals, enzymes and proteins were purchased from Sigma-Aldrich.

Neonatal (1–2 days) Wistar rats were purchased from the State University of Campinas animal facilities. After decapitation, neonatal islets were isolated by collagenase (EC 3.4.24.3) digestion of pancreata in Hanks balanced salt solution as described in Ref. [19]. Islets were washed twice in sterile Hanks solution and cultured in RPMI 1640 medium supplemented with 2 g/l $NaHCO_3$, 1% penicillin/streptomycin (Nutricell, Brazil), 10 mmol/l D-glucose, pH 7.4. Approximately 1000 islets/dish were maintained at 37 °C in humidified atmosphere with 3% CO_2 for 3 days. The medium was renewed every 24 h.

2.2. Peroxidase activity at high concentrations of H_2O_2

Islet extracts were obtained by mechanical high-speed disruption of the islets in Hanks solution at 4 °C. Homogenates were centrifuged at 10,000 × *g* for 8 min for the removal of cellular membranes and organelles. The protein concentrations of the liquid and pellet phases were measured (Biorad) and adjusted to 100 μg/ml of protein with the addition of Hanks solution. H_2O_2 was added at a final concentration of 2.3 mmol/l to each sample and its concentration was measured at 0 and 5, 10, 20, 30, 40, 60 min after the additions, using colorimetric reaction (Glucose GOD PAP-Laborlab, Brazil), against a H_2O_2 standard curve. Values were fitted with single exponential $[H_2O_2](t)=[H_2O_2]_{t=0} \times e^{-kt}$ decay curves. The peroxidase activity of each sample is represented by the *k* value (decay constant) obtained.

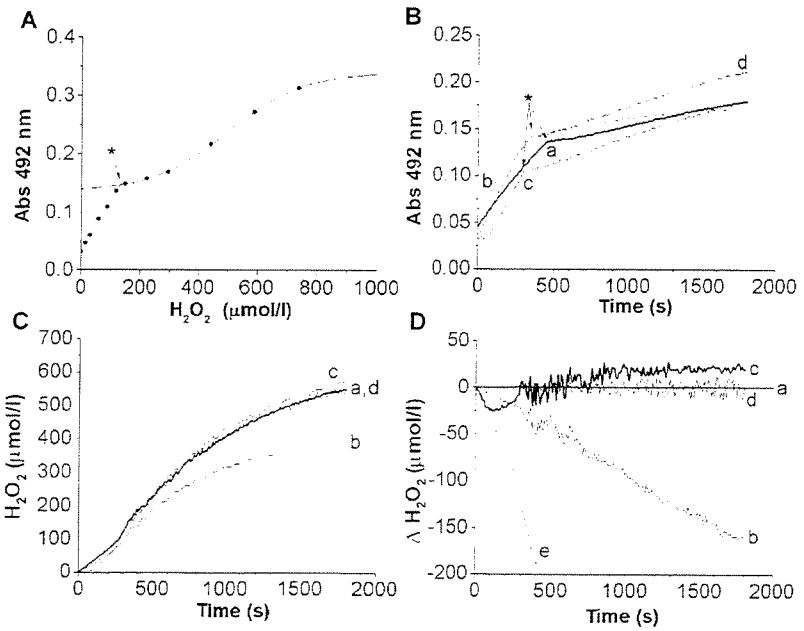

Fig. 1. Time course of H_2O_2 detection. (A) Standard curve constructed with known concentrations of H_2O_2. (B) Typical curves of H_2O_2 detection from samples containing (a) Hanks pH 7.4, (b) soluble islet extract 200 μg/ml of protein, (c) L-cysteine 50 μmol/l, and (d) cystine 25 μmol/l. (C) Detected H_2O_2 by reaction through HRP, with values obtained as described. (D) Differences between detected H_2O_2 registered by the obtained curves and the blank curve. Line (e) shows predicted values if no H_2O_2 has been processed through HRP.

2.3. Peroxidase activity at low concentrations of H_2O_2

Islet extracts were prepared as described above, followed by separation of the liquid (soluble) and the solid (insoluble) phases. The final protein concentrations were adjusted to the referred values in legends. The reaction medium was prepared by the addition of glucose GOD PAP to the solution at a final concentration of 5% (v/v), with other additions referred to in the legends. The reaction was started by the addition of 0.28 mmol/l D-glucose to the media, kept at 30 °C and with absorbances measured at 492 nm each 10 s for 1 h. At the concentration used, H_2O_2 produced from glucose by the reaction is consumed either by the sample peroxidases or by the horseradish peroxidase (HRP, EC 1.11.1.7). The last consumes H_2O_2 and 4-amino-antipydridine (4AA) to form the detected dye in such a way that a greater peroxidase activity in the sample decreases the measured absorbance. Typical detection curves are shown in Fig. 1B.

We observed different kinetics at the reaction catalyzed by HRP, as a consequence of the varying concentrations of 4AA along the experiment. The point marked with an asterisk (*) in Fig. 1A represents the modification of kinetics. Since the samples produced different initial values of absorbance, we took this point of changing kinetics as a standard for adjusting measured absorbances on the standard curve (Fig. 1A). The typical computed values of detected H_2O_2 are shown in Fig. 1C. Peroxidase activities were taken from curves plotted as shown in Fig. 1D, representing the differences between the detected H_2O_2 in the samples and in the blank (Hanks pH 7.4). The values computed were angular coefficient from the lines.

H_2O_2 concentrations in the medium were measured by the reaction between H_2O_2 and KI (1 mol/l) in Hanks supplemented with 2 mmol/l KH_2PO_4, pH 7.4, which produces a significant increase in the absorbance at 405 nm. Standard H_2O_2 curves for this assay ranged from 0 to 1 mmol/l. Aliquots from the reaction media were taken at 0, 5, 10, 20, 40 and 60 min of reaction, revealing an almost constant concentration of 5.7 ± 0.7 μmol/l H_2O_2 along the whole experiment.

We also verified that the islet extracts were unable to metabolize glucose at the experimental conditions used

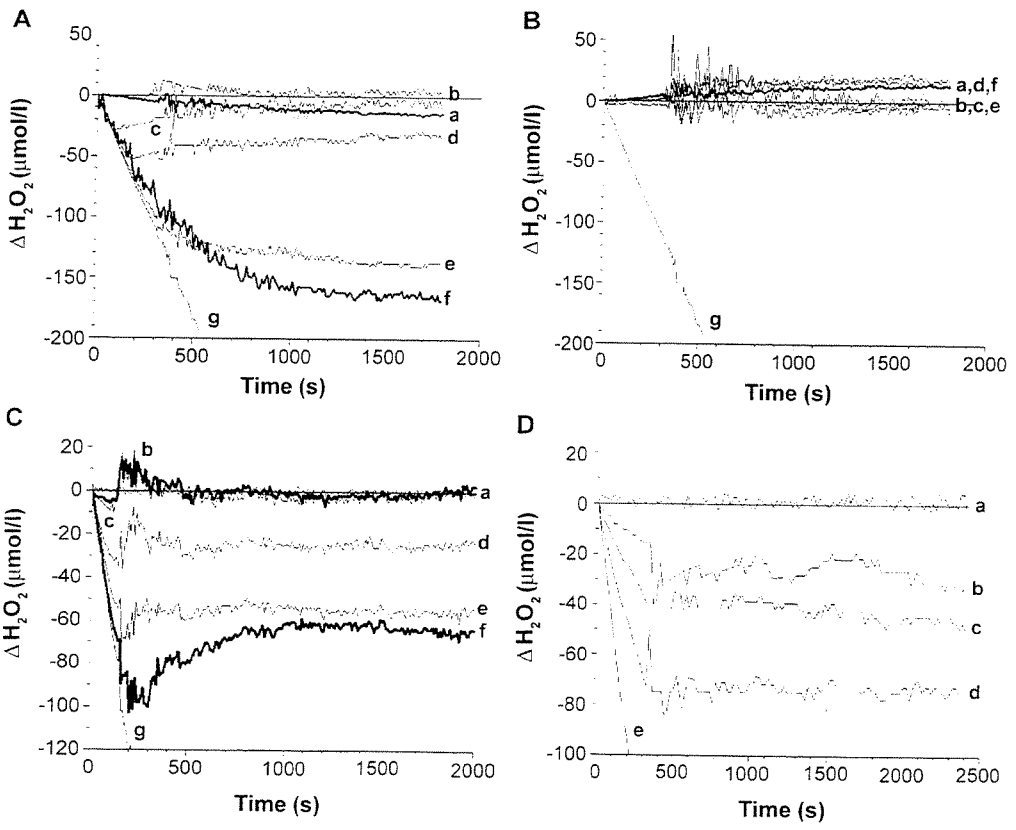

Fig. 2. Determination of the reactivity of small molecules with H_2O_2. Each reagent has been added prior to the beginning of the reaction. (A) L-cysteine was added at concentrations of (a) 0, (b) 35, (c) 70, (d) 100, (e) 140, and (f) 180 μmol/l. (B) Cystine was added at concentrations of (a) 0, (b) 20, (c) 40, (d) 60, (e) 80, and (f) 100 μmol/l. (C) 2-Mercaptoethanol was added at concentrations of (a) 0, (b) 10, (c) 25, (d) 50, and (e) 75 μmol/l. The (g) line shows the predicted values if no H_2O_2 has been detected. (D) NADPH was added at concentrations of (a) 0, (b) 20, (c) 50, and (d) 100 μmol/l. The (e) line shows the predicted values if no H_2O_2 has been detected. Each line is representative of three independent experiments and shows the difference between detected H_2O_2 in samples and in the respective blanks of each experiment (Hanks).

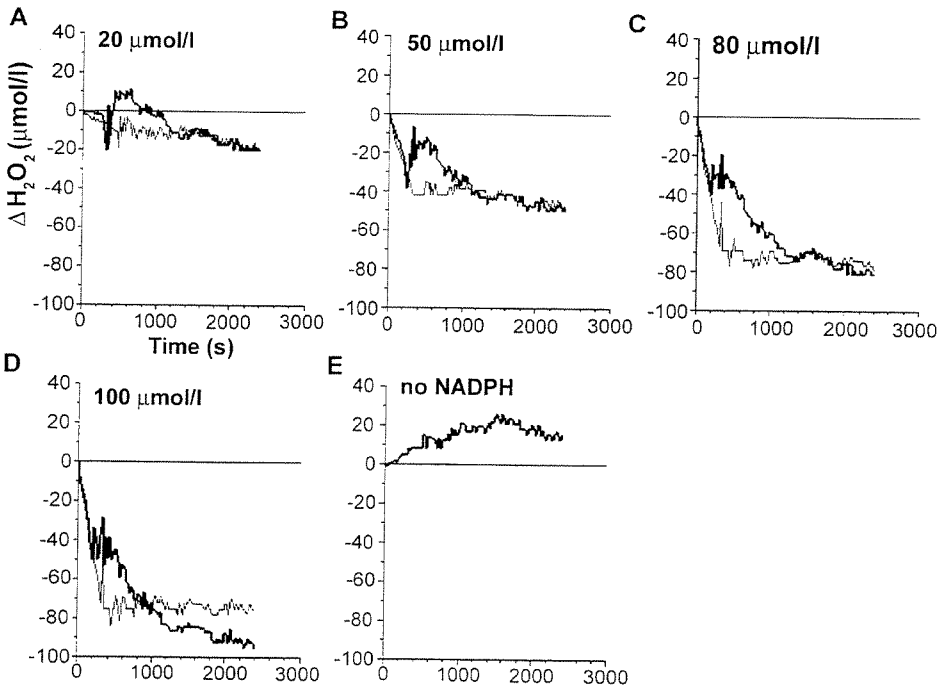

Fig. 3. Catalase activity with addition of NADPH. NADPH was added to the media without (thin lines) or with 26 μg/ml catalase (shaded lines). The concentrations of NADPH are indicated. Each line is representative of three independent experiments and shows the difference between detected H_2O_2 in samples and the respective blanks of each experiment.

(Hanks buffer, pH 7.4, 30 °C). Samples of soluble and insoluble extracts with up to 200 μg/ml of protein in Hanks pH 7.4 were incubated with 0.5 mmol/l D-glucose at 30 °C for 1 h, with no detectable variation of the glucose concentration.

2.4. Interaction between islet proteins and chemicals

In some experiments, addition of NADPH, L-cysteine (CSH), β-mercaptoethanol, dithiothreitol (DTT), cystine (CSSC), hydroxylamine, palmitate, KCN, EDTA, EGTA, *o*-phenantroline or other enzymes/proteins was done at the concentrations given in the legends. NADPH is consumed as a primary reductant by many cellular enzymes; CSH, β-mercaptoethanol and DTT are reduced thiols that can be used as reductants [20]; hydroxylamine is a catalase inhibitor; palmitate is the major fatty acid transported by serum albumin; KCN is a general inhibitor of Fe^{3+}/heme-containing enzymes; EDTA and EGTA are potent bi- and trivalent ions chelators; *o*-phenantroline is an iron-chelator. The reagents were added immediately before the start of the reaction or in the course of it, when indicated in the figures. Peroxidase activity of the extracts was taken before (sample

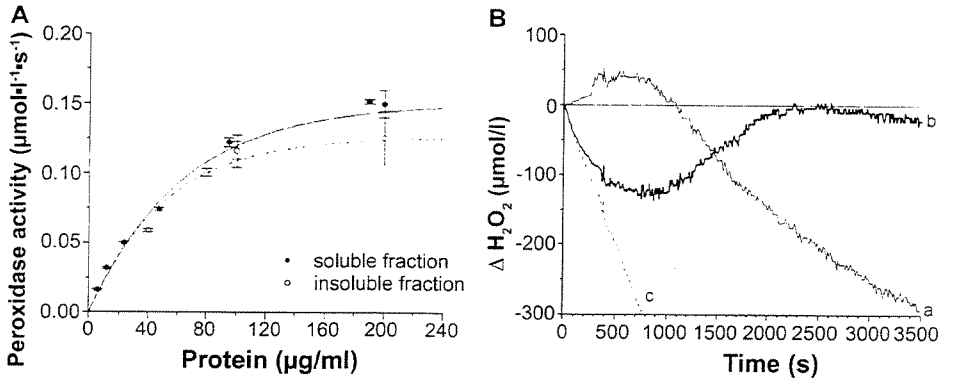

Fig. 4. Intrinsic peroxidase activity of islet extracts. (A) Activities were measured as maximal H_2O_2 degradation rate compared to blanks (Hanks alone). Each point is mean ± S.E. of four experiments. (B) Typical activity of denatured soluble extract. Samples were heated for 5 min at 100 °C and cooled to 4 °C. The lines represent: (a) soluble extracts (100 μg/ml of total protein) before denaturation, (b) soluble extracts (100 μg/ml) after denaturation, and (c) predicted values if no H_2O_2 was detected.

with no additions), during (after addition and before the slope of the curve returned to the original value) and after the consumption of the reagents. This activity is assigned to the ability of the samples to decrease the amount of quinone formed by the HRP system. In some experiments, the slope induced by the chemical was repeated two or three times, as if the reaction occurred in steps. The activities then computed were slopes kept from the first step.

Solutions of bovine serum albumin (BSA, fraction V) and pure catalase (EC 1.11.1.6) in Hanks pH 7.4 were used as models for simple protein and active peroxidase.

2.5. Statistics

Groups of evaluated peroxidase activity were compared by ANOVA, as required. The missing indicators represent samples not compared.

3. Results

3.1. Reactivity of H_2O_2 and small molecules

Aliphatic thiols such as 2-mercaptoethanol, dithiothreitol and L-cysteine are electron donors to the HRP compound I and also powerful H_2O_2 scavengers. Fig. 2A and B show the effect of different concentrations of L-cysteine and its oxidized form cystine (inactive), added immediately before the onset of the reaction. Fig. 2C shows a similar result with 2-mercaptoethanol. In these figures, the thiols completely abolished H_2O_2 detection until its end, while cystine had no effect.

Addition of NADPH also induced HRP compound I conversion to compound 2, yielding $NADP^+$ and thus $O_2^{\cdot-}$, its product by reaction with molecular oxygen [15,27]. NADPH competes with 4AA for compound I decreasing the detected amount of 492 nm colored quinone. The generated $O_2^{\cdot-}$ is an inhibitor of HRP activity.

Proteins able to trap $O_2^{\cdot-}$ (like the SOD isoforms) increase the rate of NADPH consumption and reduce the quinone production. Fig. 2D shows a decreased H_2O_2 detection due to varying concentrations of added NADPH.

3.2. Catalase activity

The K_m value for catalase is 70 ± 2 μmol l of H_2O_2 [1,15]. Fig. 3A shows that the enzyme does not have peroxidase activity at the concentrations of H_2O_2 used (5.7 ± 0.7 μmol l). When catalase is present in the medium, the addition of NADPH produced a slower reaction with compound I (Fig. 3A, B, C and D), without changing the reduced amount of hydrogen peroxide.

3.3. Intrinsic activity of islet proteins

Soluble and insoluble islet extracts showed dose-dependent and similar saturating peroxidase activity independent of any cofactor (Fig. 4A). Heat denaturation of soluble proteins also creates a peroxidase activity, although only transient, similar to that observed with pure thiols (Fig. 4B). In order to evaluate the participation of protein reduced thiols in the islet peroxidase activity, islet extracts were treated for 30 min in the dark with 100 μmol l iodoacetamide, which decreased the peroxidase activity by 28% and by 38% in soluble and insoluble extracts, respectively. These results indicated that the intrinsic peroxidase activity is partly provided by unspecific oxidation of reduced thiol groups.

Typical inhibitors of catalase were tested. Hydroxylamine caused a strong inhibition of HRP when 4AA concentration decreased, with small or no effect with a high amount of 4AA (beginning of the experiments). The specific inhibitor 3 amino 1,2,4-triazole (3AT) is oxidized by HRP compound I generating a yellowish compound whose formation kinetics did not show a neck point. Treatment of the samples with EDTA or EGTA did not modify intrinsic enzymatic activity (Fig. 5A). In addition, cyanide failed to inactivate soluble (Fig. 5B) or insoluble fraction (data not shown).

The intrinsic peroxidase activity of islet extracts were compared to plasma and hemolysed blood samples; the

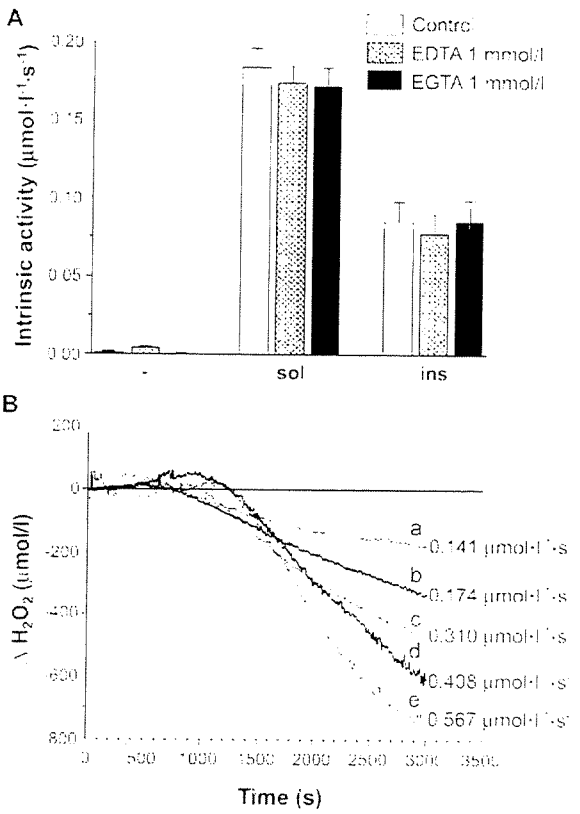

Fig. 5. Intrinsic peroxidase activities of samples exposed to chelating agents. Hanks solution, soluble (sol) or insoluble (ins) islet extracts were (A) EDTA or EGTA, (B) cyanide at concentrations of (a) 0, (b) 2, (c) 4, (d) 6, and (e) 10 mmol l. Each line is representative of four independent experiments.

values were 0.0116 ± 0.0003 and 0.140 ± 0.009 min^{-1} for high concentrations of H_2O_2, respectively. Islet extracts showed activity of 0.035 ± 0.001 min^{-1}, almost completely attributed to soluble fraction [11]. Despite the high difference in activities at high concentrations of H_2O_2, the peroxidase activity of plasma and hemolysed blood samples at low concentrations of H_2O_2 were similar (0.117 ± 0.001 $\mu mol \cdot l^{-1} \cdot s^{-1}$ for plasma and 0.120 ± 0.001 $\mu mol \cdot l^{-1} \cdot s^{-1}$ for hemolysed samples). CAT and GPx are located inside erythrocytes and, probably, contribute poorly to H_2O_2 scavenging at low concentrations of peroxide. Mixing soluble islet extract with either plasma or hemolysed blood samples (200 μg/ml of each protein) gave the same activity of plasma or hemolysed blood samples alone.

3.4. Interaction between islet proteins and thiols

Intracellular content of free thiols is up-limited at 150 μmol/l, with a partition of 50–100:1 between reduced and oxidized forms [17]. Most of reduced thiols are located at the periphery of the mitochondria [22]. We used reduced L-cysteine as a model of reduced thiol to verify if peroxidases in soluble or insoluble extracts depend on free thiols to be active. In this system, aliphatic thiols like L-cysteine are oxidized by the HRP compound 1, producing RS• radicals that may oxidize exposed thiolic groups of the proteins.

The presence of a reduced thiol (same results with 2-mercaptoethanol and dithiothreitol) induced a thiol oxidation rate to the HRP-containing media that is compatible with previous results [23]. As shown in Fig. 6A and B, thiol addition simulates a peroxidase activity (due to compound 1 conversion to compound 2) that reached its highest values with 50 μmol/l of cysteine. Otherwise, thiol addition caused little inhibition in the peroxidase activity of soluble extract but induced a powerful inhibition of insoluble extract activity (Fig. 6C and D). BSA at concentrations below 9 μmol/l dose-dependently increased CS• generation by reduction of HRP compound 1, whereas higher concentrations failed to produce higher effects.

In order to investigate if inhibition of peroxidase activity was due to formation of unspecific disulfide bonds with the protein chains or to chemical oxidation of thiols by metal ions attached to the proteins, samples were pre-treated with cyanide concentrations higher than 1 mmol/l and then exposed to L-cysteine along the experiment. Cyanide completely counteracted the inhibition of peroxidase activity by L-cysteine (Fig. 7) on soluble and insoluble samples. Otherwise, cyanide caused no peroxidase inactivation.

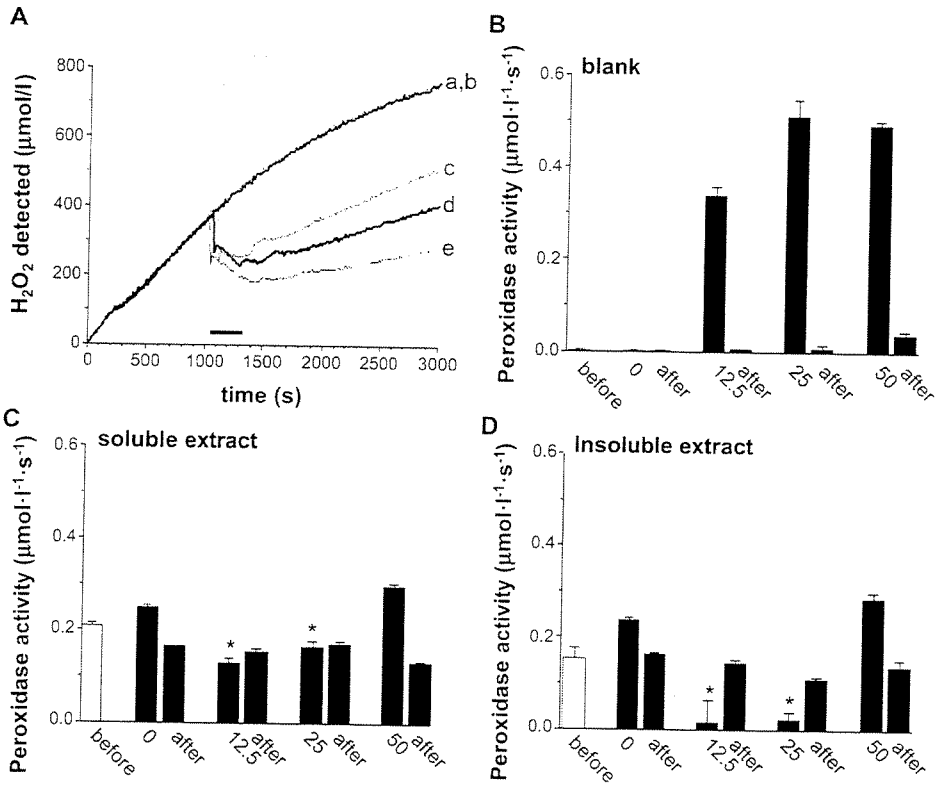

Fig. 6. Peroxidase activities of samples affected by thiol addition. (A) Typical H_2O_2 detection curves with 10 μl of L-cysteine added at $t = 1000$ s to final concentrations of (b) 0, (c) 12.5, (d) 25, and (e) 50 μmol/l. In line (a), there was no addition. (B) Peroxidase activities attributed to the medium before, during, and after cysteine consumption. Cysteine was added to the concentrations indicated (μmol/l). (C,D) Activities attributed to islet extracts (100 μg/ml of protein) before, during, and after cysteine consumption. Bars are mean ± S.E. of four experiments and represent the slopes taken immediately after the acute bending of the H_2O_2 detection lines shown in panel A. *$P < 0.05$ compared to samples with no cysteine addition.

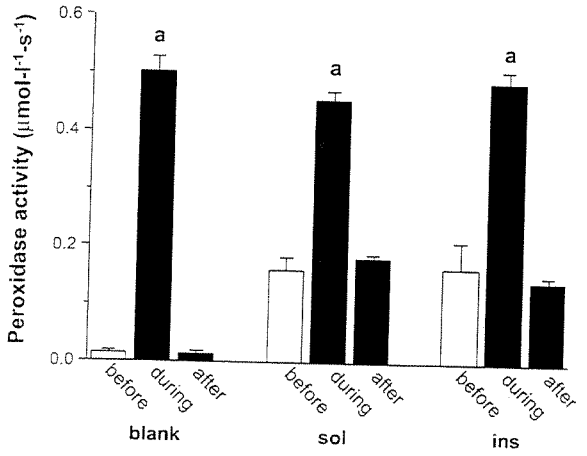

Fig. 7. Protective effect of cyanide on thiol-induced inhibition of peroxidase activity. Islet extracts were incubated for 30 min at 37 °C with at least 5 mmol/l of KCN and adjusted to final concentrations of 100 µg/ml of total protein and 1 mmol/l cyanide. Cysteine (50 µmol/l) was added at $t = 1000$ s. Bars are mean ± S.E. of four experiments and represent the slopes taken before, during and after cysteine consumption just after the acute bending of H_2O_2 detection lines shown in Fig. 6A. *$P < 0.05$ compared to samples with no cysteine addition.

3.5. Superoxide scavenging by islet proteins

Superoxide is produced through reduction of molecular oxygen and oxidation of the NADP· radical. By competition with 4AA for HRP compound 1 and inhibition of native HRP through O_2^- formation, the addition of NADPH to media during the reaction decreased the quinone production (Fig. 7A). As O_2^- production increases, the HRP cycling rate reduces and sensibility to NADPH decreases. This effect has been measured through the quinone formation rate in order to evaluate a superoxide scavenging activity by the extracts. BSA was used as a control for unspecific scavenging activity of proteins.

Fig. 8 shows that 50 µmol/l of NADPH is sufficient to saturate the NADP· production by HRP cycling. The same saturating pattern was shown by the soluble extract. Subtraction of intrinsic activities revealed that the two dependencies with NADPH concentration were identical. This dependence was highly different between blank, insoluble extract, BSA or pure catalase, which showed no saturating concentration of NADPH in the range used (Fig. 8C,D and F), probably by scavenging either NADP· or O_2^- anions formed [24].

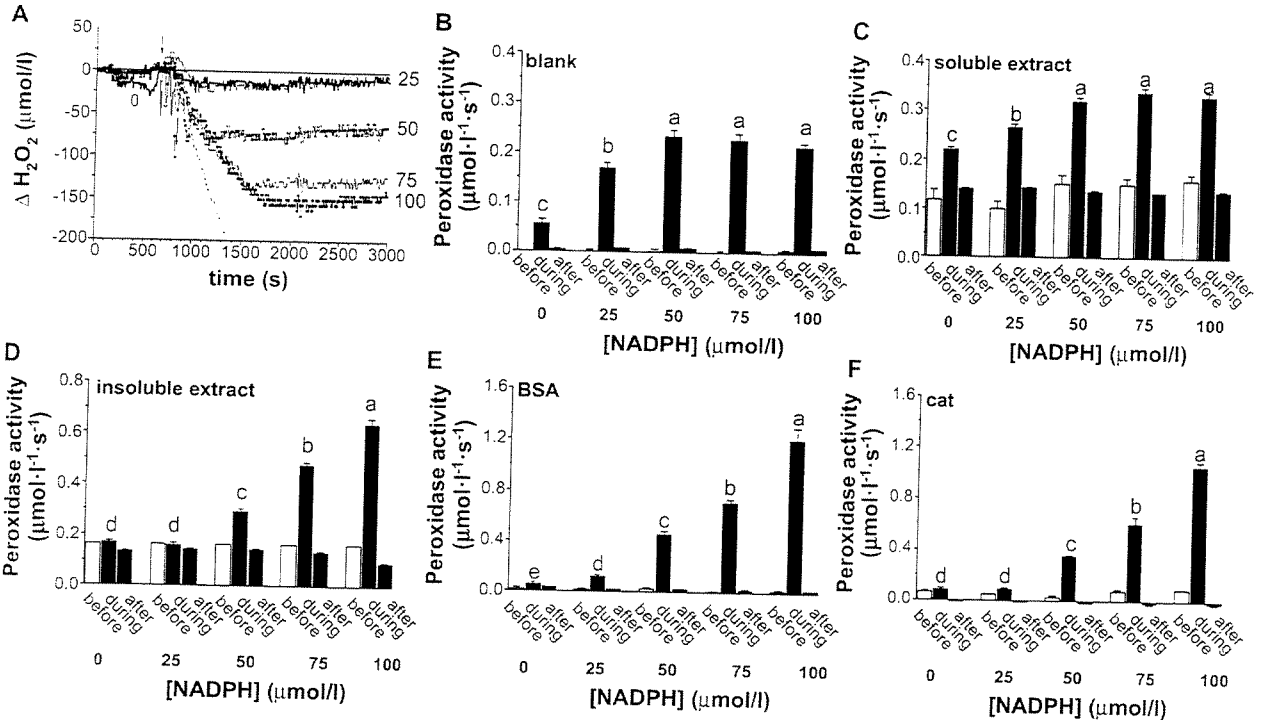

Fig. 8. Effect of NADPH addition on peroxidase activity. (A) Typical curves representing the differences between detected H_2O_2 in samples and in the blank (no NADPH). Concentrations of NADPH (arrow) in µmol/l are indicated. The dashed line represents the expected result if no H_2O_2 was detected. (B), (C), (D), (E), and (F) represent samples containing Hanks solution, soluble extract, insoluble extract, BSA, and catalase (cat), respectively. Bars are mean ± S.E. of four experiments and represent the H_2O_2 disappearance due to NADP· production. Protein concentrations in panels C, D, and E were 100 µg/ml. The concentration of catalase was 26 µg/ml. Different letters indicate significant difference between columns (*$P < 0.05$).

Increasing concentrations of BSA (0–18 μmol/l) were used as samples with 50 μmol/l NADPH added during the experiment. The $NADP^+$ production increased together with BSA concentration and saturated at 9 μmol/l BSA. A similar saturating effect was seen with CAT and insoluble extract, but not soluble extract (data not shown). Addition of palmitate to the BSA solution from 0 to 81 μmol/l did not alter the effect of BSA on $NADP^+$ production.

3.6. Factors affecting unspecific superoxide scavenging

In order to search for a superoxide scavenging activity in proteins that are not peroxidases (i.e. BSA), the metal chelators *o*-phenantroline, EDTA and EGTA were used. The soluble extract proteins at concentrations up to 500 μg/ml revealed no higher activities than those shown in Fig. 8C. BSA and insoluble extract both showed a decreasing $NADP^+$ production as the reason NADPH/protein decreases (data not shown).

O-phenantroline per se did not alter the $NADP^+$ production (Fig. 9A). Soluble (Fig. 9B), but not insoluble (Fig. 9D) extract and BSA (Fig. 9C) superoxide scavenging activities were diminished by *o*-phenantroline. This suggests that soluble extract and BSA (but not insoluble extract) are partially dependent on $Cu^{2+}/Fe^{3+}/Mn^{3+}$ ions natively attached to sample proteins to exert superoxide scavenging activity. EDTA and EGTA (1 mmol/l) added to the reaction failed to affect $NADP^+$ generation by any of the samples (data not shown).

Treatment of the samples with 1 mmol/l cyanide caused no inhibition of $NADP^+$ production, reinforcing that superoxide scavenging is not due to heme $Cu^{2+}/Fe^{3+}/Mn^{3+}$ ions, but to natively attached ones (data not shown).

4. Discussion

The redox state of pancreatic B cells is a critical point for determining their survival or death through activation of apoptotic/necrotic pathways. We have previously shown that islets possess catalase activity, although very little if compared to liver (1%), blood plasma (33%) or hemolysed samples (8%). This catalase activity reduces progressively along the lifetime with islets from adult rats having only 20% of that observed for neonatal islets [1]. In a situation of acute and severe oxidative stress, catalase activity may be necessary (but not sufficient) for islet cell survival.

At low H_2O_2 concentrations (5.7 ± 0.7 μmol/l), catalase has no other detectable activity than to slow $NADP^+$ production by HRP compound I (Fig. 3). Since the amount of H_2O_2 consumed by HRP cycling is the same, with or without catalase, a partition of NADPH between HRP and catalase may occur, resulting in the same loss of H_2O_2 detection. Through catalase reaction, the NADPH-consuming stage may be slower (but not negligible) than through HRP compound I. In a H_2O_2-rich medium, catalase compound I is readily re-converted to ferricatalase through a two-electron reaction with H_2O_2. However, in a H_2O_2-poor

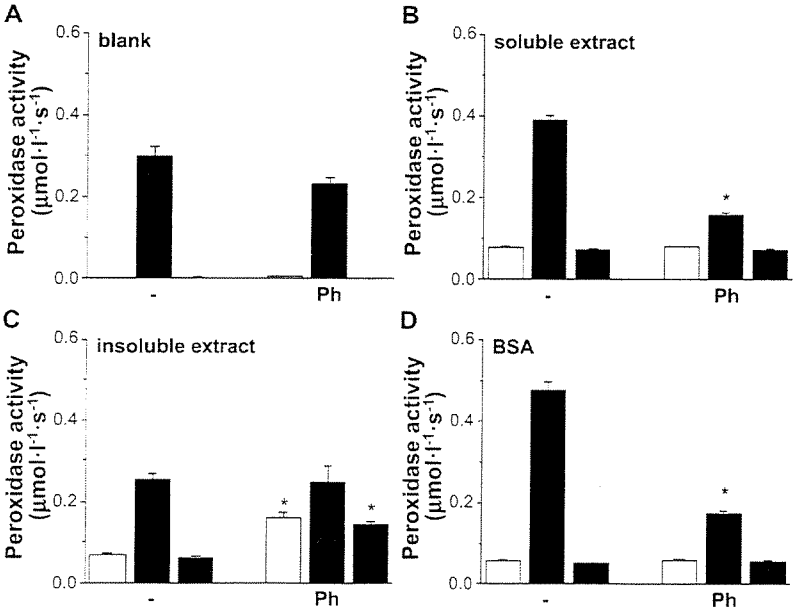

Fig. 9. Effect of *o*-phenantroline on $NADP^+$ production. NADPH (50 μmol/l) was added at $t = 600$ s. Bars are mean ± S.E. of four experiments and represent the H_2O_2 disappearance due to $NADP^+$ production before (white bars), during, and after NADPH consumption. In panel A, no protein was added. In panels B, C, and D, protein concentrations were adjusted to 200 μg/ml. Phenantroline (1 mmol/l) was added before the start of the experiments. Significant differences between values obtained without (–) and with phentroline (Ph) are indicated (*$P < 0.05$).

medium, catalase compound 1 scavenges superoxide and is not inactivated at conversion to compound 2 only if there is a reductant such as NAD(P)H, abundant in the medium (Fig. 8F) [14,24].

We have observed that samples with highly different catalase activities have similar peroxidase activities, suggesting a marginal participation of catalase in the intrinsic peroxidase activity of the samples. When islet extracts were mixed with other protein sources (such as plasma), in a medium containing both low and high peroxidase components, the second one is responsible for H_2O_2 degradation. Thus, if catalase, which has low peroxidase activity, is present in the islet fractions used, it is not responsible for any H_2O_2 degradation. Finally, concentrations of catalase several times higher than that found in pancreatic islets would be necessary in order to generate the intrinsic peroxidase activity observed in soluble and insoluble fractions.

As catalase, BSA and the insoluble fraction of islets showed similar behavior towards $NADP^{\bullet}$ generation. This suggests a non-enzymatic mechanism of relevant superoxide scavenging by these proteins (Fig. 8D and E), which is not valid for the soluble fraction. BSA is known to accommodate $Cu^{2+}/Fe^{3+}/Mn^{3+}$ ions (from the solution) attached to its molecules [25]. In fact, BSA-increased generation of $NADP^{\bullet}$ is sensitive to *o*-phenantroline, but not to EDTA and EGTA. At the concentration of 100 μmol/l NADPH, when $NADP^{\bullet}$ generation by HRP compound 1 is saturated, increasing concentration of BSA dose-dependently diminished the $NADP^{\bullet}$ production, indicating that BSA per se is able to chelate or oxidize NADPH.

If metal ions participate in some $O_2^{\bullet-}$ scavenger mechanisms, the failure of EDTA, EGTA and *o*-phenantroline to reduce the insoluble fraction intrinsic peroxidase activity and superoxide scavenging supports the fact that they are not loosely attached to the protein molecules (Figs. 5A and 9C). Cyanide also failed to inhibit the peroxidase activity of insoluble fraction and its ability to remove $NADP^{\bullet}$ products, like $O_2^{\bullet-}$ anion. These results suggest that, probably, the intrinsic peroxidase activity and superoxide scavenging activity are due to Fe^{3+}/Mn^{3+} (but not Cu^{2+} ions), which may not be located inside heme structures [26–28]. Therefore, we cannot exclude the participation of catalase, at least in the superoxide scavenging, by insoluble fraction. At a high NAD(P)H disposability and superoxide production, as we may find at glucose-stimulated beta cell activity, the presence of catalase offers an alternative mechanism for protection against H_2O_2 products even if catalase is unable to decompose H_2O_2 itself [29,30].

Incubation of the samples with the thiol oxidant iodoacetamide inactivated the free (proteic) thiolic groups that may be sacrificed in unspecific oxidation by H_2O_2. This corresponds, partly, to intrinsic peroxidase activity of both soluble (28%) and insoluble islet fractions (38%), which is transiently increased several times by heat denaturation. Inside the cell, these sacrificial thiolic groups may be recurrently oxidized and reduced by reductants such as NAD(P)H, thioredoxin and glutathione. Depletion of these reductants would lead to slow but complete oxidation of the sacrificial thiolic groups, decreasing the peroxidase activity of islet cells at cytoplasm, nucleus and organelles. Such a sacrificial tool is also found in blood plasma in the form of BSA [31].

However, the exposure of proteins of the insoluble fraction to small thiolic molecules promptly inactivated them, during cysteine oxidation, in a reaction that is prevented by cyanide pre-treatment (Fig. 7). During this reaction, HRP compound 1 is not reduced by cysteine. The high rate of thiol oxidation by the insoluble fraction would suggest that this extract became rich in sulfenic acid groups (RSO^{-}), as a consequence of exposure to H_2O_2. Although at a very low magnitude, a similar effect is reported to happen with human serum albumin [20,32]. However, the inactivation of the extract is reversible and was completely prevented by cyanide, something that is incompatible with inactivation by mixed disulfide formation. If cysteine is otherwise oxidized by Fe^{3+}/Mn^{3+} ions linked to insoluble fraction peroxidases, the peroxidases may be converted to a temporary inactive form according to the following mechanism:

$$\mathrm{ins[metal^{3+}](active) + CSH \Rightarrow ins[metal^{2+}](inactive) + CS^{\bullet}} \quad (1)$$

$$\mathrm{CS^{\bullet} + H_2O_2 \Rightarrow CSO^{\bullet} + H_2O} \quad (2A)$$

$$\mathrm{CSO^{\bullet} + H^{+} + CSH \Rightarrow CSSC + H_2O} \quad (2B)$$

$$\mathrm{ins[metal^{2+}](inactive) + O_2 \Rightarrow ins[metal^{3+}](active) + O_2^{\bullet-}} \quad (3)$$

These reactions with unspecific peroxidase substrates have been previously characterized on HRP [23]. Reactions where thiol reductants are oxidized by peroxidases are very unlikely to happen in the cell since they deplete the thiol reductant (SH), inactivate the peroxidases at the insoluble fraction, and also generate superoxide radicals. An increase of small thiolic compounds inside the cell may cause oxidative stress. In addition, it was reported that high doses of cysteine, but not glutathione, led to apoptotic/necrotic destruction of beta cells [33,34]. Glutathione and other charged thiols are known to be poor electron donors to unspecific peroxidases [23].

While not affected by cyanide (Fig. 5B), the peroxidase activity of the soluble fraction is less inhibited by cysteine (Fig. 6C) than the insoluble fraction and has very little superoxide scavenger activity (Fig. 8C). The ability of *o*-phenantroline to reduce $NADP^{\bullet}$ production in the reaction media containing the soluble fraction indicates that this fraction has some similarities to BSA, with $Cu^{2+}/Fe^{3+}/Mn^{3+}$ ions weakly attached to protein molecules (Fig. 7B). However, these ions are not involved with the intrinsic peroxidase activity of this fraction.

5. Conclusions

Inside the islet cells, the intrinsic peroxidase activity of both soluble (28% sacrificial thiols) and insoluble (38% sacrificial thiols) fractions (both not heme-related and Cu^{2+} independent) may cooperate to reduce H_2O_2 concentration outside the mitochondria. If these sacrificial thiols are not reduced by NAD(P)H-dependent reductases such as glutathione reductase, it may slowly reach its end and thus deprive the cells from nearly 30% of its peroxidase activity, which is not attributable to catalase. The metal ions from the insoluble fraction can be reduced by L-cysteine to give inactive peroxidases. However, if H_2O_2 is converted to O_2^- through Fenton reactions [10], thiol oxidation [20] or any other mechanism, then superoxide anions may be scavenged almost by putative "superoxidases" at the organelle and nuclear fractions. However, if H_2O_2 is converted to O_2^- through Fenton reactions, superoxide anions may be scavenged almost by "superoxidases" at the organelle and nucleus fraction. These scavengers have metal ions strongly attached to the protein molecules but are also not heme-related and Cu^{2+} independent, probably MnSOD and/or similar enzymes. However, in islets, catalase is located almost at the soluble fraction [1] and does not have a similar behavior toward superoxide scavenging if compared to the pure enzyme. Thus, these results suggest that neither the intrinsic activity nor superoxide scavenging activity in neonatal islet cells are dependent on the catalase, but on MnSOD (or a similar enzyme) content of this tissue.

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Trabalho nº 3

ALTERED NAD(P)H PRODUCTION IN NEONATAL RAT ISLETS RESISTANT TO H_2O_2

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Key words: glucose oxidation, H_2O_2, islets, NADH shuttles, neonatal pancreatic islets, pentose phosphate pathway

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Abstract

Oxidative stress contributes to the loss of glucose-induced insulin secretion (GSIS) during the onset of diabetes mellitus. Although immature rat islets have reduced GSIS compared to mature ones, they adapt better to oxidative stress and are a good model to understand the causes that lead destruction or survival of islet cells. In this work, we cultured isolated neonatal rat islets for four days in medium containing 5.6 and 20 mmol glucose/l with or without H_2O_2 (200 μm/l) and analyzed several parameters that help the islets survive in the different media. High glucose was used since it protects neonatal islets against loss of GSIS. While none of the culture conditions increased NAD(P)H reduction rate, the islets resistant to H_2O_2 and those submitted to high glucose showed higher use of the pentose phosphate pathway and increased ATP synthesis from glucose. The GSIS in immature islets relies on NADH shuttle activities, with little contribution of reduced equivalents from the tri-carboxylic acid cycle (TCA). While high glucose concentrations increased islet mitochondrial activity, the oxidation of endogenous substrates in mitochondria together with the PPP utilization was crucial for the resistance against oxidative stress.

1. Introduction

Malfunction of pancreatic islets impairs glucose homeostasis and can lead diabetes mellitus. Insulin secretion occurs after increase of the ATP/ADP ratio in β-cells via both Ca^{2+} dependent and independent pathways. The Ca^{2+}-dependent insulin secretion is named the triggering pathway, while Ca^{2+}-independent secretion accounts for the amplifying pathway [1]. In both human and animal models, a combination of chronic hyperglycemia, high fatty acid concentration [2], cytokines and reactive oxygen species reduce the ability of the islets in responding to glucose by affecting both pathways. These pathways respond positively to cellular changes induced by high glucose such as; increase of ATP/ADP ratio and NAD(P)H concentration, increase in UCP2 activity, and augmentation of basal cytoplasmic Ca^{2+} [3].

Glycolisis-derived NAD(P)H has been implicated as a highly necessary trigger of insulin secretion [4]. NAD(P)H is transported to mitochondria matrix by NADH shuttles as a necessary complement for pyruvate-derived NADH/$FADH_2$ [5]. The transport of NAD(P)H to mitochondria by the NADH shuttle system accounts for 50% of TCA cycle activity.

In rat islets, a counter-current mechanism exports NAD(P)H from mitochondria, provided mainly by the pyr/mal shuttle. In this shuttle, pyruvate carboxylase introduces glycolisis-derived pyruvate in the TCA cycle as oxalacetate, what is converted to malate by the mitochondrial malate dehydrogenase. Malate is exported in large amounts by mitochondria of mouse and rat islets [6] and is a marker of the TCA cycle activity [7]. In rat islets, malate is promptly converted to pyruvate by the malic enzyme, which keep high concentrations of pyruvate and NAD(P)H in β-cells [2]. The citrate/isocitrate shuttle may not play a role in insulin secretion, since no insulin secretagoges provokes exportation of isocitrate from rat mitochondria [7].

Islets cultured in the presence of high concentrations of glucose, but not L-leucine and other secretagoges, show augmented sensitivity to glucose, higher insulin secretion, and resistance to deleterious effect of H_2O_2 [8, 9]. Despite insulin secretion, induced by glucose or L-leucine is similar, L-leucine alters the concentrations of metabolites in the cytoplasm of β-cells, lowering glutamate and increasing aspartate levels by allosteric activation of mitochondrial glutamate dehydrogenase [10]. Apparently, rat islets have a glutamate content enough to maintain insulin secretion for 2-3 hours through glutamate dehydrogenase and malate-aspartate shuttle activities. However, the chronic use of non-pyruvate deriving metabolites impairs the shuttle activity by depleting aspartate from cytoplasm [7, 9]. The activity of these NADH shuttles in islets is amplified several times when islets evolve from a low-responsiveness fetal state to an adult condition [11].

Recently, we have suggested that islets have a different mechanism of defense against reactive oxygen species by offering high concentrations of NAD(P)H to a enzyme-poor system [12]. Since cytoplasmic NAD(P)H must increase in order to trigger GSIS, we investigated if a process that requires NAD(P)H (such as the defense status against low concentrations of H_2O_2) is a sufficient condition to increase cytoplasmic NAD(P)H production and at the same time enhance glucose-stimulated insulin secretion.

2. Material and Methods

2.1. Chemicals

Radiochemicals were from G.E. Heath Care, aminotransferase inhibitor amino-oxyacetate (carboxymethoxylamine) was from Sigma-Aldrich, MTS/PMS preparation was from CellTitter96 aqueous assay (Promega) and all RT-PCR reagents from Invitrogen. Other reagents were from Sigma, whenever specified.

2.1. Islets isolation and culture

Neonatal (1-2 days old) Wistar rats were from the State University of Campinas animal facilities. After decapitation, the islets were isolated by collagenase (EC 3.4.24.3) digestion of pancreata in Hanks balanced salt solution (in mmol/l: 137 NaCl, 5.5 KCl, 4.5 $NaHCO_3$, 0.4 KH_2PO_4, 0.4 Na_2HPO_4, 0.8 $MgSO_4$, 1.5 $CaCl_2$, pH 7.4). Islets were extensively washed in sterile Hanks solution and cultured in RPMI 1640 medium supplemented with 2g/l $NaHCO_3$, 1% (v/v) penicillin/streptomycin, 10 mmol/l D-glucose, pH 7.4. Approximately 1000 islets/dish were maintained at 37°C in humidified atmosphere with 3% CO_2 for 2 days before further additions. The culture media contained 5.6 mmol/l or 20 mmol/l of glucose, with or without 200 µmol/l H_2O_2, for additional 4 days. The medium was renewed each 24 h. Islet experimental groups were assigned according culture conditions: G5.6 (islets cultured in 5.6 mmol/l of glucose), P5.6 (5.6 mmol/l of glucose and 200 µmol/l H_2O_2), G20 (20 mmol/l of glucose) and P20 (20 mmol/l of glucose and 200 µmol/l of H_2O_2).

2.2. Insulin secretion

Batches of 10 islets each were incubated for 30 min in 37°C Krebs-Hepes buffered saline (KHBS, in mmol/l: 115 NaCl, 10 $NaHCO_3$, 5 KCl, 1 $MgCl_2$, 2.5 $CaCl_2$, 15 Hepes) containing 0.5 g/l BSA and 5.6 mmol/l of glucose, pH 7.4 and equilibrated with 95% O_2 and 5% CO_2. The medium was discarded and the islets incubated for a further period of 1 h in 1 ml of KHBS containing 2.8 or 16.7 mmol/l glucose with the additions described in legends. The supernatant was collected and insulin was measured by radioimmunoassay.

2.3. Glucose uptake and metabolism

Batches of 50 islets each were incubated for 2 h at 37°C in KHBS containing 2.8 or 16.7 mmol/l glucose with trace amounts of either D-[U-^{14}C]glucose or D-[1-^{14}C]glucose for measuring $^{14}CO_2$ production. The batches were added with HCl 1N to stop respiration and the $^{14}CO_2$ collected for 1h in 4 °C NaOH 1N solution. For glucose uptake assays, the batches were incubated in similar conditions with 2-deoxy-D-[U-^{14}C]glucose for 2 h. Then, the islets were washed twice with 4 °C KHBS, sedimented and homogenized with addition of Trizol (Invitrogen). Partition of glucose between TCA cycle and pentose phosphate pathway (PPP) were calculated stating that total $^{14}CO_2$ produced from labeled glucose were from TCA cycle and the PPP. Naming G1 the D-[1-^{14}C]glucose metabolized and GU the D-[U-^{14}C]glucose metabolized in simultaneous measurements, for each experiment we calculated as (6×G1-GU)/(6-1/6) the glucose passed through the TCA cycle and (6×GU-G1)/(6-1/6) the glucose passed through the PPP.

2.4. RT-PCR

Groups of 1000 islets were homogenized in Trizol following phenol-chloroform RNA extraction according manufacturer's instructions. RNA integrity was asserted through agarose-gel separation. One to five µg of total RNA was transferred to reactions vials with 1 µg dNTP mix. The reactions were incubated for 5 min at 65°C before addition of 150 ng random primers, for 10 min at 25°C before addition of 14.3 mmol/l $MgCl_2$, 2.8 mmol/l DTT, 0.4 U/µl RNase-out and at 42°C for 2 min before addition of 1.25 U/µl RNA Super Script II. Samples were incubated at 42°C for 50 min, at 70°C for 15 min, and then cooled to 4°C. The obtained cDNAs were diluted in PCR buffer (60 mmol/l Tris-HCl, 1.5 mmol/l $MgCl_2$, 15 mmol/l NH_4SO_4, pH10) with 50 mmol/l $MgCl_2$, 0.3 mmol/l each of dATP, dCTP, dGTP and dTTP, 2.5 U/µl Taq DNA polymerase (Gibco/BRL), then added 10 mmol/l

forward primer and reverse primer. PCR amplification of cDNA was done with a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The PCR program involved the following cycle profile: 32 cycles of denaturation for 1 min at 94°C, annealing for 1 min, extension for 1.5 min at 72°C, and maximization of strand completion for 7 min at 72°C. The annealing temperatures and the number of cycles used in each amplification are shown in legends. Following amplification, the cDNA fragments were analyzed on 1.6 % agarose gels containing a 100 bp DNA molecular weight ladder (Gibco/BRL). PCR products were analyzed by ethidium bromide UV fluorescence in a Gel Doc EQ analyzer (Bio-Rad).

Internal control of reactions was chosen among various used controls, to which the RPS-29 subunit of small ribosomal unit 40S showed the best homogeneity between treated and non-treated groups. Primers were designed and tested against *Rattus norvergicus* genome (Gene Bank) to ensure no amplification of other cDNAs. The oligonucleotide primers were as follows: **RPS-29 (forward)** 5'-AGGCAAGATGGGTCACCAGC-3'; **RPS-29 (reverse)** 5'-AGTCGAATCCATTCAGGTCG-3'; **rat pro-insulin 2 (forward)** 5'-TTGCAGTAGTTCTCCAGTT-3'; **rat pro-insulin 2 (reverse)** 5'-ATTGTTCCAACATGGCCCTGT-3'; **GLUT2 (forward)** 5'-CATTGCTGGAAGCGTATCAG-3'; **GLUT2 (reverse)** 5'-GAGACCTTCTGCTCAGTCGACG-3'; **mitochondrial pyruvate dehydrogenase subunit E1 alpha 2 (mPDH) (forward)** 5'-TCAAGTACTACAGGATGATG-3'; **mPDH (reverse)** 5'-GGCGTACATGTGCATTGATC-3'; **mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH) (forward)** 5'-AGAAAGTCTGCATCGTAGGCT-3'; **mGPDH (reverse)** 5'-GGAAGTTGGGTGTTTGCATCA-3'; **mitochondrial malate dehydrogenase (mMDH) (forward)** 5'-CCTGAAGCCATGATTTGCATC-3'; **mMDH (reverse)** 5'-TTCTTGATGGAGGCTTTCAGC-3'; **glucose-6-phosphate dehydrogenase (G6PDH) (forward)** 5'-AGCTCCAATCAACTGTCGAAC-3'; **G6PDH (reverse)** 5'-TCCTCAGGGTTGAAGAACATG-3'. All annealing temperatures and number of cycles were chosen to agree maximal sensibility to sample cDNA content.

2.5. NAD(P)H determination

NAD(P)H was measured by the coupled reduction of phenazine methosulfate (PMS) and subsequent transfer of electrons to the tetrazolium salt MTS, both membrane permeable. Mixing of MTS and PMS solutions was done according to the manufacturer instructions.

Little change of 492 nm absorbance was seen with concentrations of NAD(P)H below 10 μmol/l. Due to an important interference of proteins bound to NAD(P)H, standard curves were less accurate in low concentrations of NAD(P)H. However, by spectroscopic analysis was observed an increase of ~10^{-3} cm^{-1} isl^{-1} light absorption in 650 nm when different number of islets where disrupted in MTS/PMS solution containing 2 mg/ml of BSA. Heat-denaturated islet homogenates were used as negative controls.

Static measurements of NAD(P)H were done by incubating groups of 200 islets in KHBS containing 2.8 or 16.7 mmol/l of glucose, reproducing the same conditions used in insulin secretion experiments. Islets were then washed in ice-cold Hanks solution and immediately sonicated in 150 μl of Hanks solution. Homogenates were centrifuged 10,000g × 2 min to remove islet debris. Supernatants were then added with MTS/PMS solution and incubated for 30 min at room temperature before recording absorbance in 650 and 405 nm (background). Samples with no islet were used as blanks. In each experiment, 650 nm absorbance changes were normalized to the mean absorbance of G5.6 samples incubated in 16.7 mmol/l of glucose.

Dynamic measurements of NAD(P)H were done by incubating groups of 20 islets in 200 μl of KHBS containing 0 or 20 mmol/l of glucose and 5% (v/v) of MTS/PMS. This concentration of MTS/PMS was chosen after testing concentrations from 1 to 20% (v/v) and searching for best sensitivity. As judged by a transient rate of MTS/PMS reduction, concentrations of PMS/MTS above

7% caused cell death due to NAD(P)H depletion. Samples were incubated for 3 h under 95% O_2 + 5% CO_2 atmosphere (140 μmol/l of O_2 in KHBS), then changed to a 100% N_2 atmosphere (90 μmol/l of O_2) for other 3 h to cause hypoxia. Part of the samples were returned to the 95% O_2 atmosphere and cell death, due to hypoxia, was estimated to be less then 20%. Samples with no islet were used as blanks and 492 nm absorbance values were recorded each 30 min. NADPH standard curves were used to calculate the reduced amounts of NAD(P)H in samples. The NAD(P)H reduction rate (NRR) values were taken from the temporal increase of MTS/PMS absorbance in each sample.

2.6. NAD(P)H reduction rate partitioning

Calculations of the NAD(P)H reduction rate partition were done on NRR from each sample by supposing NRR to be composed of four independent components: cytoplasmic NRR from endogenous sources (endo.cyto), cytoplasmic NRR from glucose oxidation (gluc.cyto), mitochondria NRR from endogenous sources (endo.mito) and mitochondria NRR from glucose-provided metabolite oxidation (gluc.mito). We assumed that the mitochondrial components depend on adequate oxygen supply, while cytoplasmic ones are essentially anaerobic. The NRR from endogenous sources was estimated from values observed in absence of glucose. These endogenous fuels were presumed to be mostly fatty acid and amino acids.

2.7. Statistics

Point-to-point comparisons were done by Students t-test. Groups were compared by two-way ANOVA using unpaired Tukey-Kramer method as post-test. Results were considered significantly different if $P<0.05$. In RT-PCR experiments, results were considered different only if $P<0.001$.

3. Results

At the end of the culture period, GSIS from control group G5.6 was similar to that observed from fresh isolated islets (not shown). Islets cultured with high glucose or H_2O_2 showed increased glucose oxidation and higher GSIS compared with controls (Fig. 1), but reduced coupling of GSIS to glucose oxidation: in ng secreted insulin / pmol of total metabolized glucose (16.7 mmol/l glucose) G5.6 1.4 ± 0.2^{a}, P5.6 0.5 ± 0.1^{b}, G20 0.7 ± 0.2^{b} e P20 1.1 ± 0.2^{ab} (different letters indicate $P<0.05$). This suggests that glucose and H_2O_2 treatments could share a mechanism that deviates carbons from ATP-production pathway.

Despite a significant loss of islets in culture with H_2O_2, treatments with high glucose or H_2O_2 caused no change in GLUT2 mRNA content (Fig. 2B) or decrease in glucose uptake (Fig. 2A). In addition in the post-culture islet population, there was no significant change of insulin content (data not shown). Both high glucose and H_2O_2 treatments selected islets with augmented oxidation of glucose through the PPP (Fig. 1B), that produces cytoplasmic NADPH. This is consistent with an increased expression of G6PDH mRNA (Fig. 3A). Genes encoding proteins related to NAD(P)H transit between mitochondria and cytoplasm also had their expressions modified by glucose and H_2O_2 (Fig 3B and C): mGPDH expression was increased in high glucose-treated islets, while mMDH expression was higher in islets resistant to H_2O_2. The mPDH expression was not affected by high glucose or H_2O_2 treatments (Fig. 3D).

When challenged with glucose, islets resistant to H_2O_2 showed less dependence of glucose oxidation upon the mal/asp shuttle, as shown by the use of the transaminase inhibitor amino-oxyacetate (Fig. 4A). Remarkably, the participation of the mal/asp shuttle in glucose oxidation was activated by a glucose challenge only in P5.6 group, while it was required for GSIS in all groups (Fig. 4B).

To induce insulin release, NADH from the TCA cycle and cytoplasm must enter the respiratory chain and donate electrons to ATP synthesis. Electrons from NADH are donated to complex I or directly to complex II, through the activity of $FADH_2$-linked glycerol-phosphate shuttle. Inhibiting the complex I with rotenone, we observed that high-glucose and H_2O_2 showed synergic effects in increasing the use of mitochondrial NADH for GSIS, although these effects were not additive (Fig. 5). IC_{50} values obtained on GSIS (glucose raising from 2.8 to 16.7 mmol/l) were G5.6 43 ± 1^a, P5.6 29 ± 1^b, G20 20 ± 2^c, P20 18 ± 1^c nmol/l of rotenone (different letters indicate $P<0.05$).

To further understand the NAD(P)H reduction inside the islets, we performed time-coursed and static determinations of NAD(P)H; where the islets were incubated with no glucose (to estimate the NAD(P)H reduction rate (NRR) from endogenous sources), and/or to inhibit mitochondrial activity (hypoxia). In a similar series of experiments, islets were incubated with 200 μmol/l of H_2O_2 to localize inhibition/activation of NAD(P)H reduction pathways.

In figure 6 it is evident that most of islet NAD(P)H comes from endogenous fuels. The islets from G20 group, the most glucose-responsive, when challenged with 20 mmol/l glucose, doubled the NRR keeping constant the NAD(P)H contents (Fig. 6E). Other groups increased NRR in about 25-50% when islets were exposed to the same concentration of glucose. Only in islets cultured with 5.6 mmol/l of glucose was observed an increase in NAD(P)H content in response to glucose stimulation (Fig. 6E). This indicates a poor transference of NADH electrons to the respiratory chain. Moreover, both high glucose and H_2O_2 treatments increased aerobic contribution to NRR. In all groups of islets there small stimulation by glucose on NRR under hypoxia (Fig. 6A, B, C and D).

The results shown in figure 6 were used to estimate cytoplasmic and mitochondrial NRRs, provided from endogenous sources (fatty acids, amino acids, etc) or from glucose oxidation. It was evident that the contribution of endogenous fuels to NRR differentiated islets of the poor glucose-

responsive group G5.6 and the high glucose-responsive groups G20 and P20 (Fig. 7A). Only in group G20 islets was observed a high NRR induced by glucose, in similar to what is reported to happen in adult islets. This glucose-responsive NRR was abolished when group G20 islets were incubated with 200 μmol/l of H_2O_2 (Fig. 7A).

Islets from groups G5.6 and G20 incubated for 3h in 200 μmol/l of H_2O_2 reversibly decreased the NRR from endogenous fuels and from glucose. However, the incubation with H_2O_2 increased the cytoplasmic glucose-derived NRR in group G5.6 islets that was not observed in long-time H_2O_2 islets from group P5.6 (Fig. 7B).

It is noteworthy that the incubation with H_2O_2 quite abolished the mitochondrial glucose-derived NRR in islets from all groups, except group P20. A similar H_2O_2 inhibition of mitochondrial activity is since long ago reported to happen in mature islets when exposed to the oxidant.

4. Discussion

In this work, we attempted to trace the mechanism by which low concentrations of H_2O_2 select islets with increased GSIS, and how this mechanism is shared with high glucose treatment. A critical coupling between glucose oxidation and insulin secretion by rat β-cells arises from cytoplasmic NAD(P)H introduced in the mitochondria matrix by the three NADH shuttles, the pyr/mal, glycerol-phosphate and mal/asp shuttles. In fact, glucose-responsiveness of islets is thought to be triggered by the NAD(P)H generated in cytoplasm [4]. Although mature islets show glucose oxidation and GSIS highly connected, it is clear that inhibiting the mal/asp shuttle with AOA in some groups inhibited glucose oxidation, but not insulin secretion in neonatal islets (Fig. 4).

Islets from adult rats also show very small glucose utilization through the pentose phosphate pathway [13], and NADPH in the cytoplasm comes mostly by pyr/mal and mal/asp shuttles [14].

Glycerol-phosphate and mal/asp shuttles activity also increase Ca^{2+} oscillation frequency in cytoplasm of glucose-stimulated islets [15] and trigger Ca^{2+} entry in mitochondria to activate three Ca^{2+}-dependent dehydrogenases of the TCA cycle [16]. Differing from mature ones, culture-selected islets from neonatal rats maid strong use of the PPP, increasing its activity in adaptation to high glucose (2×) and H_2O_2 (3×), without enhancing the glucose flux trough TCA cycle (Fig. 1B). Combined with the increased mal/asp shuttle activity (Fig. 3C), it makes possible that, unlike in mature islets, the PPP provide NADPH as fuel for ATP production, concomitantly generating the backbone for nicotinamide synthesis. Late results from Weinhaus *et al.* also have shown that fetal and neonatal islets are not deficient in mitochondrial fating of metabolites, but exhibits poorly processing trough glycolisis [17].

There are evidences that the pyr/mal shuttle is highly active in rat islets and exports malate from mitochondria, removing reduced equivalents from mitochondrial matrix when β-cells are stimulated by glucose. So, the shuttle does not contribute for insulin secretion unless the NADH could re-enter the mitochondria [7]. The mal/asp and glycerol-phosphate shuttles insert NADH into mitochondria matrix or directly at complex II of the respiratory chain, respectively being inhibited by rotenone and antimicyn A [5]. Control islets (G5.6) displayed high dependence of glucose oxidation and GSIS upon the mal/asp shuttle (Fig. 4), but this was not true for other groups. The increased expression of mMDH in islets cultured with H_2O_2 paralleled the high shuttle participation in GSIS, but not in glucose oxidation. In neonatal islets, the apparent paradox reinforced the hypothesis that GSIS is mainteined by ATP generated mainly from cytoplasmic NAD(P)H, not requiring the full oxidation of glucose in TCA cycle. This became more evident when hypoxia provoked only a marginal effect on NRR of the less glucose-responsive G5.6 group (Fig. 6A).

Islets were selected along culture period with necrotic and apoptotic pathways leading to destruction of islets unable to counteract damage imposed by H_2O_2 and/or high glucose concentrations

[8]. Although it should be expected higher NRR in islets that survived oxidative stress (like in P5.6 and P20 groups), the observed NRRs were not different in islets cultured with H_2O_2. In these islets, increase in NRR due to stimulation with 20 mmol/l glucose also did not indicate depletion of endogenous substrates, despite the heterogeneity in GSIS (Figs. 7B and D). The variation of NAD(P)H content, observed only in islets cultured with 5.6 mmol/l glucose, suggests that these islets used a minor fraction of reduced equivalents for ATP generation, perhaps because of this being less responsive to glucose (Fig. 5). A significant part of these equivalents are thought to sustain repair mechanisms like thiols reduction [14].

From literature, it appears that β-cell mitochondria have a limited capacity of condensing oxalacetate and acetyl-CoA to form citrate, thus increasing several times the malate concentration inside the matrix when secretagoges provide carbon for the TCA cycle [7]. This malate export from mitochondria and the pyr/mal shuttle can provide a production of cytoplasmic NAD(P)H to be inserted in mitochondria by the other shuttles. It is reported that exposure of adult islets to the complex I inhibitor rotenone doubled the formation of non-superoxide radicals from glyceraldehyde, while exposure to the complex II inhibitor myxothiazol had no effect [18]. Altogether, this indicates that mature islets usually make much less use of the electron insertion at the level of complex II than at complex I, even when glycerol-phosphate shuttle activity is favored. Culturing immature neonatal islets with 20 mmol/l glucose, the mGPDH expression was increased by 2-3 fold compared with controls (5.6 mmol/l glucose) (Fig. 3B). As a consequence, both glucose oxidation and insulin release became less sensitive to inhibition of the mal/asp shuttle with AOA (Fig. 4B), suggesting a higher use of the glycerol-phosphate shuttle in neonatal than in mature islets. In late works, glycerol-phosphate shuttle activity was associated to GSIS by modulating the mitochondria activity, its deficiency being observed in models of type 2 diabetes [19].

While culture with high glucose concentrations did not modify NRR from endogenous substrates, nor significantly modified the glucose-responsiveness of NRR (Fig. 6C and D), it increased the islet basal content of reduced NAD(P)H (Fig. 6E) in sufficient amount to strength coupling between NAD(P)H and ATP productions (Fig. 5). It is noteworthy that islets cultured with H_2O_2 or high glucose showed increased oxidation of glucose in the repair process of the pentose phosphate pathway (Fig. 1B).

Islet maintained in the presence of 20 mmol/l glucose generates, in culture, more NAD(P)H derived from glucose than controls (G5.6 group). By using this extra NAD(P)H into repair mechanism, it is acceptable that only islets cultured in high glucose kept the necessary environment to maintain glucose-responsiveness when exposed to H_2O_2 (Fig. 1A).

Comparing the calculated NRR from endogenous sources and from glucose oxidation, we observed that although islets cultured with high glucose showed higher generation of reduced species in mitochondria, this was not required to present high GSIS. On the contrary, islets with high secretory glucose-responsiveness distributed NRR both in cytoplasm and mitochondria and showed slightly higher non-mitochondrial glucose metabolism, that may be linked to the observed PPP activity (Fig. 7A).

The NAD(P)H content in islets may also be linked to glucose-responsivess by being required for lipid syhthesis [2], a process that may happen in islets cultured with 20 mmol/l of glucose. Acyl-Coa synthesis alters the pyr/mal shuttle activity, being necessary for GSIS [5, 20]. Although the maximal NRR observed in islets stimulated with 20 mmol/l glucose did not correlate with GSIS, the good correlation with glucose oxidation rate reinforces that NADH shuttles could limit the carbon flux into TCA cycle of neonatal islets (Fig. 7A, upper table and Fig. 4A).

Figure 7B shows that even islets cultured in media containing H_2O_2 presented inhibition of NRR (or augmented consumption of NAD(P)H) when lately exposed to H_2O_2. Characterized by little

effect of H_2O_2 on the NRR provided by endogenous fuels and high inhibition of glucose-derived NRR, this inhibition may indicate a quite selective effect of H_2O_2 on processing of glucose inside β-cells, although not restricted to cytossolic or mitochondrial pathways. The more severe effect of H_2O_2 was seen on group G20 islets when first exposed to H_2O_2 (Fig 7B), leading perhaps to the low glucose-responsive NRR in mitochondria of group P20 islets, despite this may not accomplish maximal GSIS (Fig. 1A). Keeping in mind the high glucose-sensible processing of glucose derivates in mature islets mitochondria [21], this suggests that islets develop a high GSIS through increasing mitochondrial processing of glucose derivatives that is simultaneously too sensible to oxidative stress and may be easily damaged by H_2O_2.

First exposure of islets cultured in physiologic concentrations to H_2O_2 evoked a glucose-dependent rise in cytoplasmic NRR. The islets may be able to resist acute oxidative stress if it occurs concomitantly with a rise in cytoplasmic metabolism of glucose. Chronic exposure of islets to high glucose concentrations may generate oxidative stress (see Fig. 1B, G20), so the activation of PPP and cytoplasmic glucose-dependent NRR are characteristic features of islets that resist to H_2O_2 (Fig. 7B). The activation of cytoplasmic glucose-dependent NRR is a suitable mechanism for preserving glucose-responsiveness of the pancreatic islets.

Finally, in addition to generating NADPH to protect islets from oxidative stress, our results suggest that immature islets from neonatal rats have the plasticity to modify the site and the source of NAD(P)H reduction, changing glucose oxidation rate and the coupling with ATP production. Islets cultured at low concentrations of glucose are unable to keep high contents of NAD(P)H. These islets increase the mal/asp shuttle activity to generate ATP from reduced equivalents in cytoplasm, whereas islets cultured in high concentrations of glucose generate ATP from both cytoplasmic and mitochondrial sources possibly through the glycerol-phosphate shuttle, presenting increased GSIS.

Consumption of endogenous substrates through aerobic pathways parallels a higher mitochondrial activity, coupling tight NAD(P)H reduction and ATP synthesis.

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Figure Legends

Fig. 1. (A) Insulin secretion of islets cultured 4d with 5.6 or 20 mmol/l of glucose (**G5.6** and **G20**), without or with 200 μmol/l H_2O_2 (**P5.6** and **P20**). Islets were pre-incubated 30 min. in KHBS with 5.6 mmol/l glucose, as described, then incubated either with 2.8 or 16.7 mmol/l of glucose. Bars are mean ± S.E.M. of 8 independent experiments, where different letters indicate $P<0.05$. **(B)** Partition of metabolized glucose between TCA cycle (open part of bars) and the PPP (filled bars). Values were calculated from $^{14}CO_2$ produced by D-[U-^{14}C]glucose and D-[1-^{14}C]glucose metabolism in vials containing KHBS with 2.8 or 16.7 mmol/l of glucose, as described. All samples showed similar results on 2.8 mmol/l condition. Bars are mean ± S.E.M. of 8 experiments performed simultaneously with D-[U-^{14}C]glucose and D-[1-^{14}C]glucose. Different letters indicate $P<0.05$ in comparisons intra TCA cycle values and intra PPP values of glucose metabolism.

Fig. 2. (A) Glucose uptake in islets cultured at the same conditions as in Figure 1. Islets were incubated 120 min. in KHBS with 2.8 or 16.7 mmol/l of glucose containing equal amounts of 2-deoxy-D-[U-^{14}C]glucose, then washed and homogenized with Trizol. Bars are mean ± S.E.M. of at least 8 experiments, where different letters indicate $P<0.05$. **(B)** Effect of culture with high glucose / H_2O_2 on islet GLUT2 mRNA levels. Annealing temperatures and cycle numbers were 55 ^{o}C, 29 cycles (GLUT2) and 57 ^{o}C, 29 cycles (RPS-29). Bars are mean ± S.E.M. of 12 experiments. Different letters indicate $P<0.01$.

Fig. 3. Effect of culture with high glucose and H_2O_2 in mRNA levels of **(A)** G6PDH, **(B)** mG3PDH, **(C)** mMDH and **(D)** mPDH. RT-PCRs annealing temperatures and cycle numbers used were 57 ^{o}C, 31 cycles (G6PDH and mG3PDH), 57 ^{o}C, 35 cycles (mMDH) and 55 ^{o}C, 32 cycles (mPDH). RPS-29

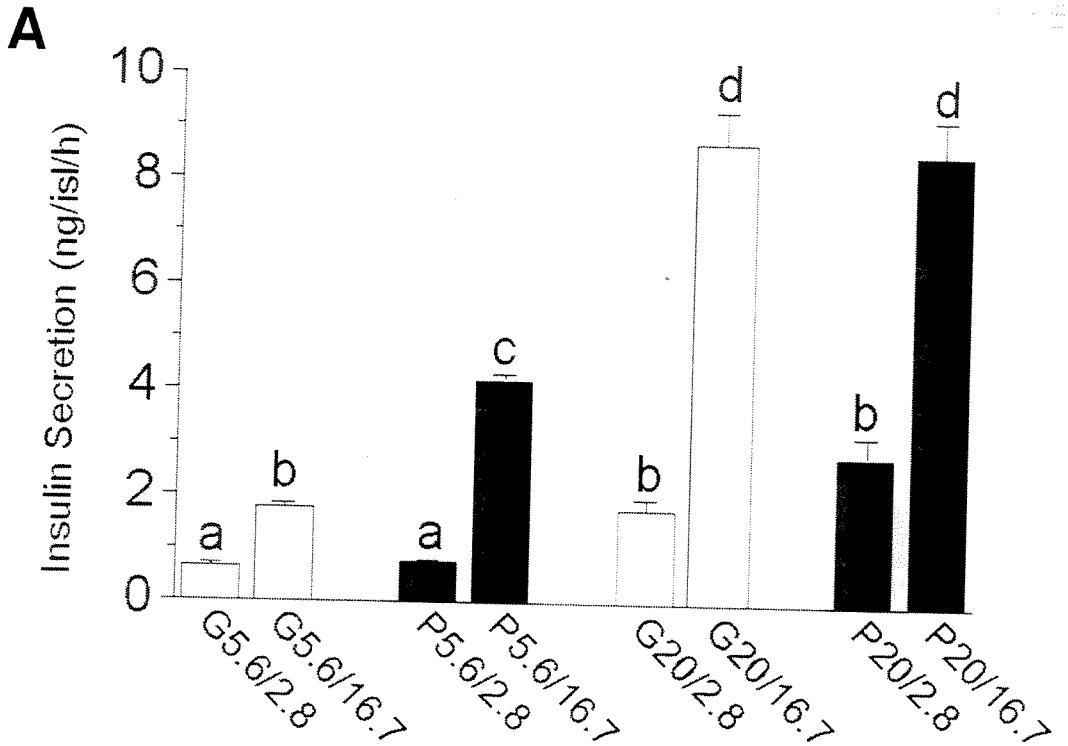
cDNA was amplified using 57 ^{o}C and 29 cycles, showing no variation among the conditions tested. Plotted columns are mean ± S.E.M. of 12 experiments. Different letters indicate P<0.001.

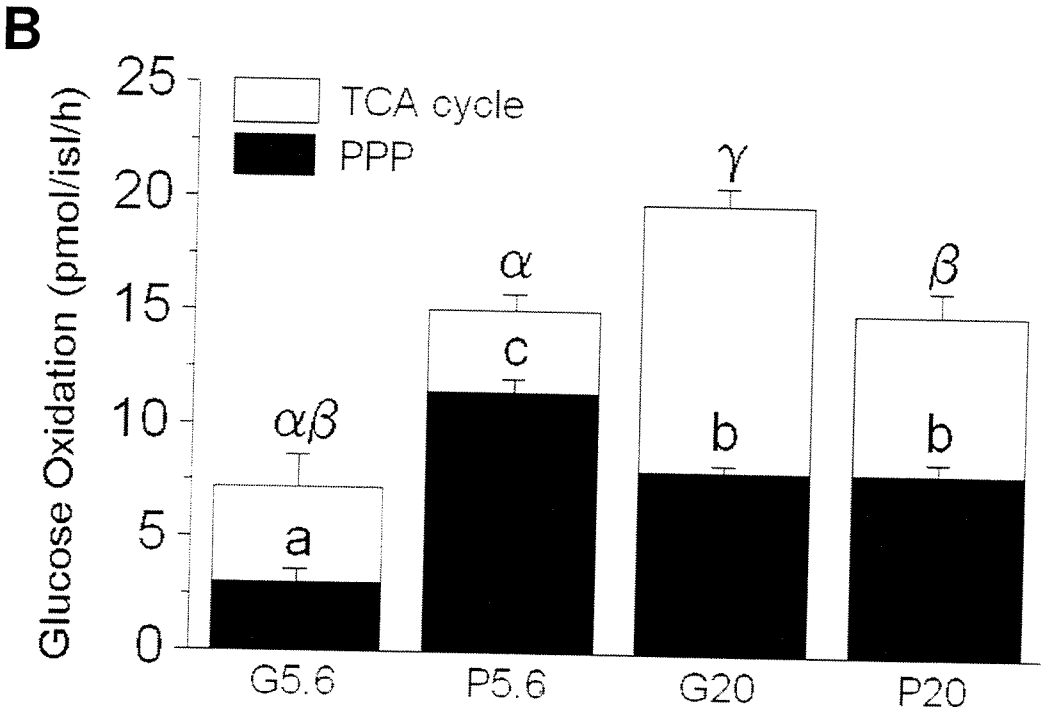
Fig. 4. (A) Glucose metabolism in islets exposed to 5 mmol/l of the aminotransferase inhibitor AOA. Islets were incubated in KHBS with 2.8 or 16.7 mmol/l glucose containing D-[U-^{14}C]glucose, as described. **(B)** Insulin secretion of islets incubated 60 min. in KHBS with 2.8 or 16.7 mmol/l glucose and 5 mmol/l AOA. Values were normalized to values found in control (no AOA) experiments realized simultaneously. Bars are mean ± S.E.M. of 12 experiments. Different letters indicate P<0.05.

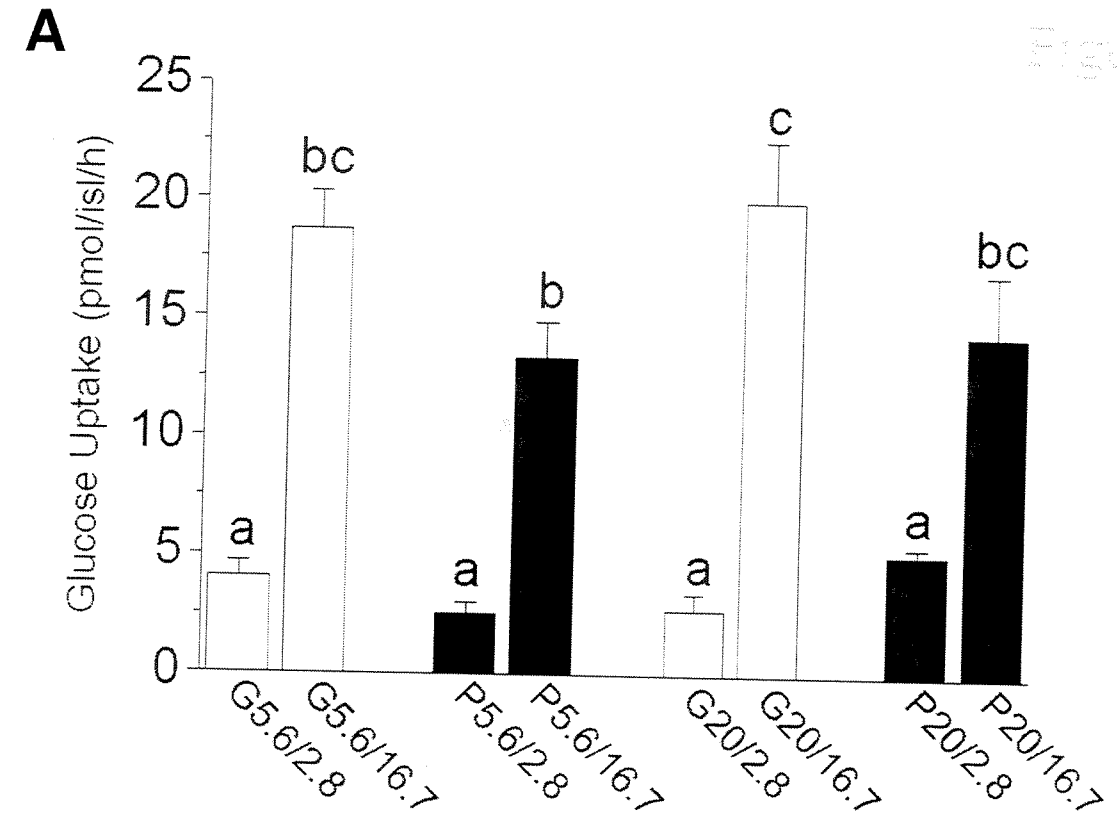
Fig. 5. Inhibition of GSIS by rotenone. Islets were incubated in KHBS buffer containing 2.8 or 16.7 mmol/l of glucose and different concentrations of rotenone. Values are the fold increase in insulin secretion when glucose concentration is raised from 2.8 to 16.7 mmol/l. Each point represents mean ± S.E.M. of 8 independent experiments. Different letters indicate P<0.05 between the entire curves.

Fig. 6. (A, B, C and D) NAD(P)H reduction rate. Islets were incubated for 3h in KHBS containing 5% (v/v) of MTS/PMS and 0 mmol/l of glucose (G0), 20 mmol/l of glucose (**G20**), 0 mmol/l of glucose + 200 μmol/l of H_2O_2 (H0) and 20 mmol/l of glucose + 200 μmol/l of H_2O_2 (H20). Incubations were performed in a 95% O_2 atmosphere that produced 140 μmol/l of O_2 in KHBS solution, then changed to a 100% N_2 atmosphere that reduced O_2 concentration in solution to 90 μmol/l. Under hypoxia (hypox), islets were incubated for another 3h. NRR of each sample was calculated as the temporal chance of NAD(P)H causing reduction MTS/PMS. Values are mean ± SEM of 8 experiments. **(E)** NAD(P)H content of entire islets incubated for 1h in KHBS containing 2.8 or 16.7 mmol/l of glucose. Values are mean ± S.E.M. of 10 experiments. Different letters indicate P<0.05.

Fig. 7. NAD(P)H reduction from endogenous fuels in cytoplasm (**endo.cyto**), from endogenous fuels in mitochondria (**endo.mito**), from glucose in cytoplasm (**gluc.cyto**) or from glucose-derived fuels in mitochondria (**gluc.mito**). Calculations were done as described, using values from Fig. 6 from islets incubated without **(A)** or with 200 μmol/l of H_2O_2 **(B)**. Total values of NRR presented in upper tables are the sum of the four fractions of each group. Statistical analyses were done only between islet culture groups. Different letters indicate $P<0.05$.


A
Insulin Secretion (ng/isl/h)
0
2
4
6
8
10
a
b
a
c
b
d
b
d
G5.6/2.8
G5.6/16.7
P5.6/2.8
P5.6/16.7
G20/2.8
G20/16.7
P20/2.8
P20/16.7


B
Glucose Oxidation (pmol/isl/h)
TCA cycle
PPP
0
5
10
15
20
25
αβ
a
α
c
γ
b
β
b
G5.6
P5.6
G20
P20


A
Glucose Uptake (pmol/isl/h)
25
20
15
10
5
0
a
bc
a
b
a
c
a
bc
G5.6/2.8
G5.6/16.7
P5.6/2.8
P5.6/16.7
G20/2.8
G20/16.7
P20/2.8
P20/16.7

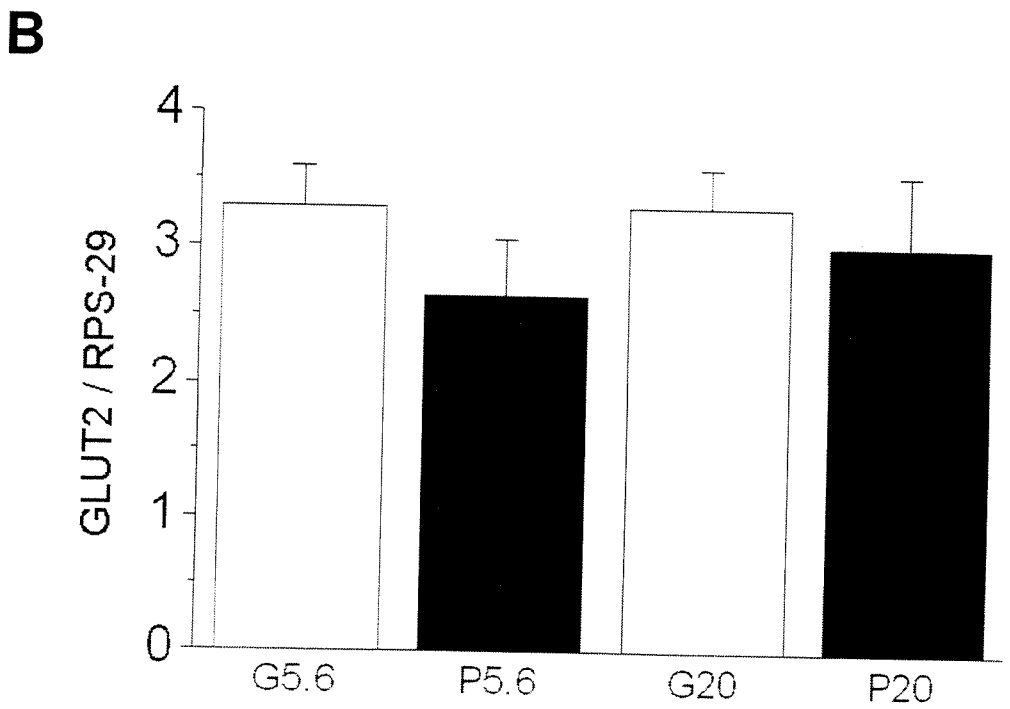

B
GLUT2 / RPS-29
4
3
2
1
0
G5.6
P5.6
G20
P20

Figure 3

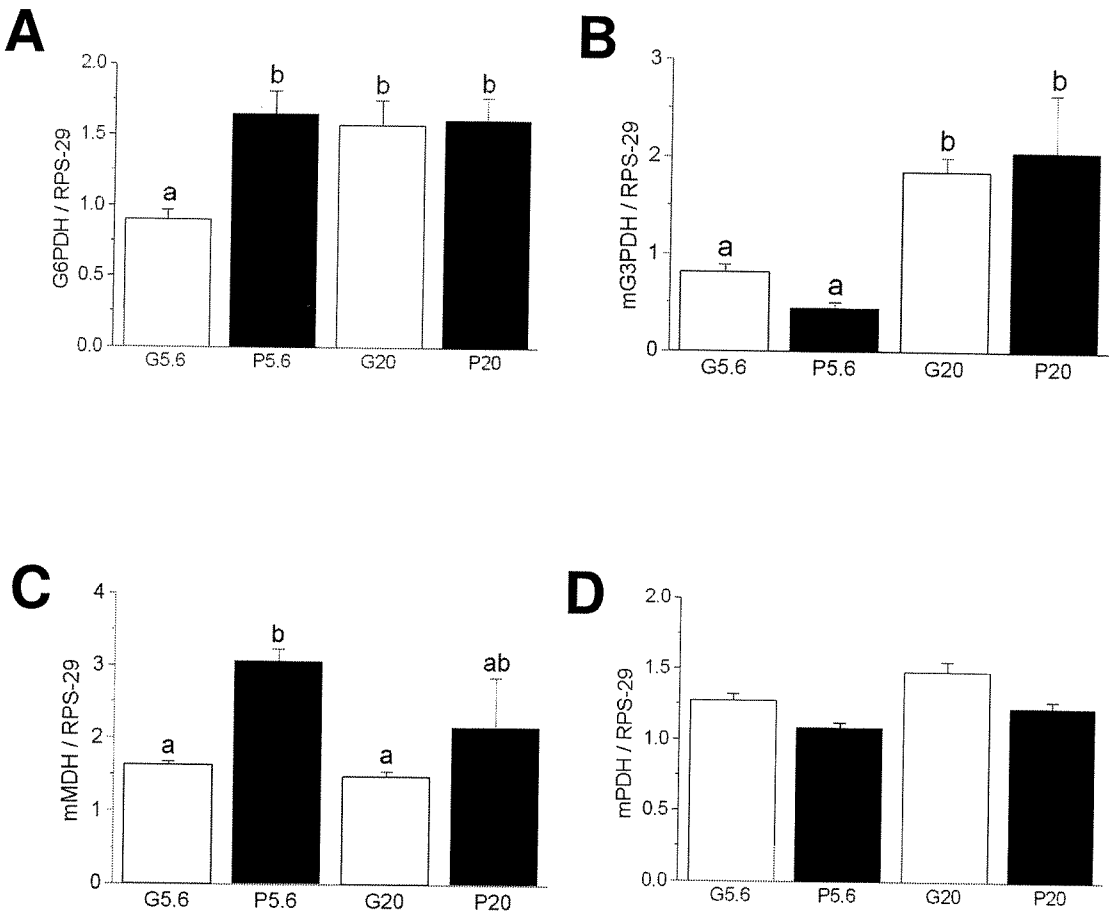

A
G6PDH / RPS-29
2.0
1.5
1.0
0.5
0.0
a
b
b
b
G5.6
P5.6
G20
P20
B
mG3PDH / RPS-29
3
2
1
0
a
a
b
b
G5.6
P5.6
G20
P20
C
mMDH / RPS-29
4
3
2
1
0
a
b
a
ab
G5.6
P5.6
G20
P20
D
mPDH / RPS-29
2.0
1.5
1.0
0.5
0.0
G5.6
P5.6
G20
P20

Figure 4

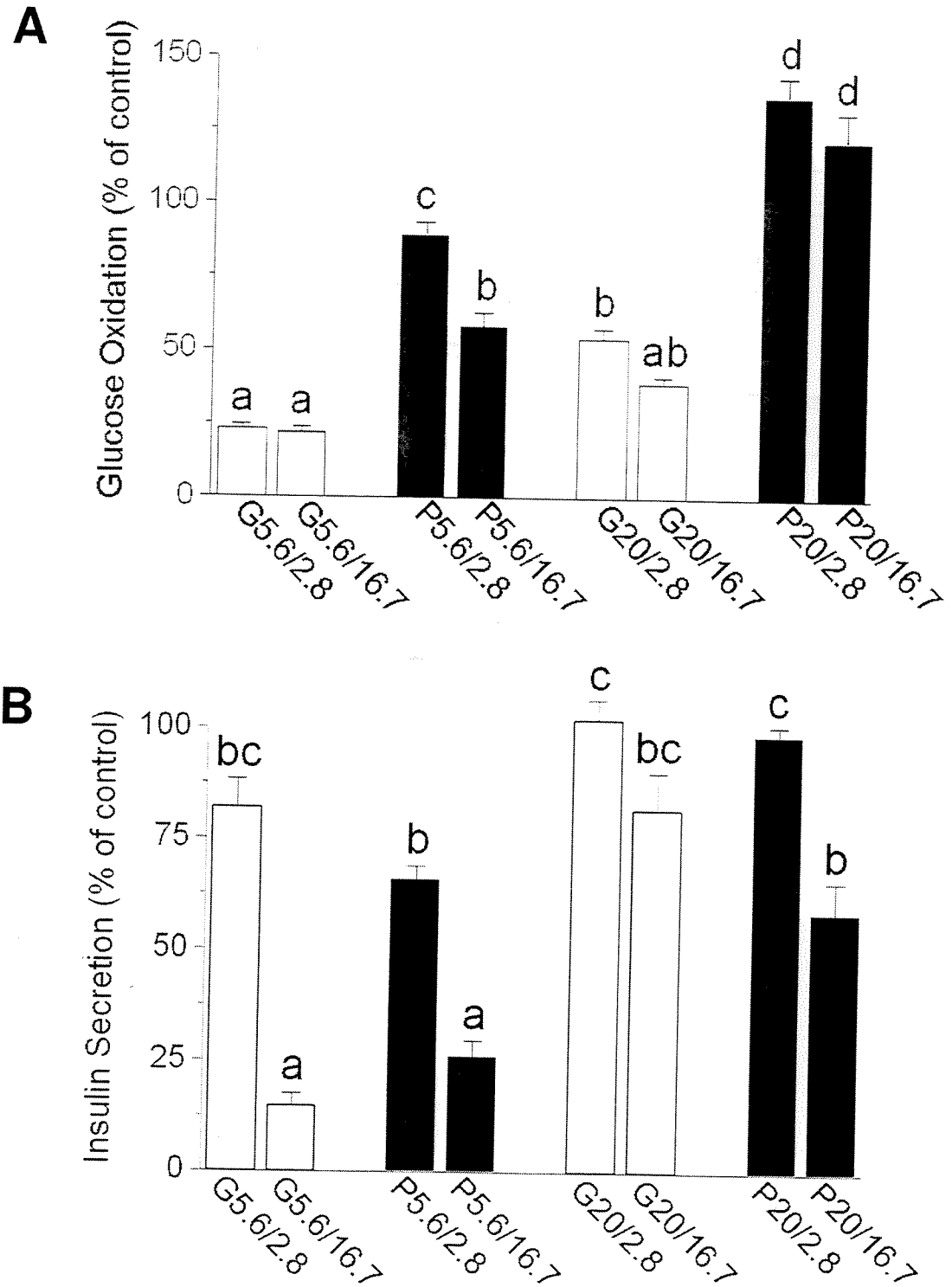
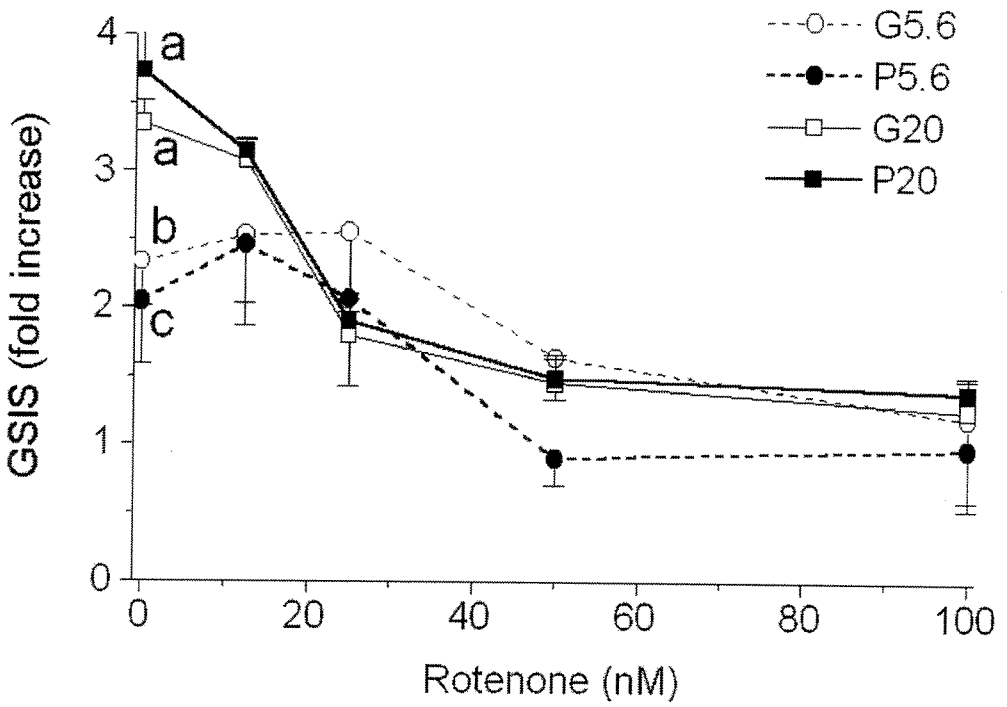
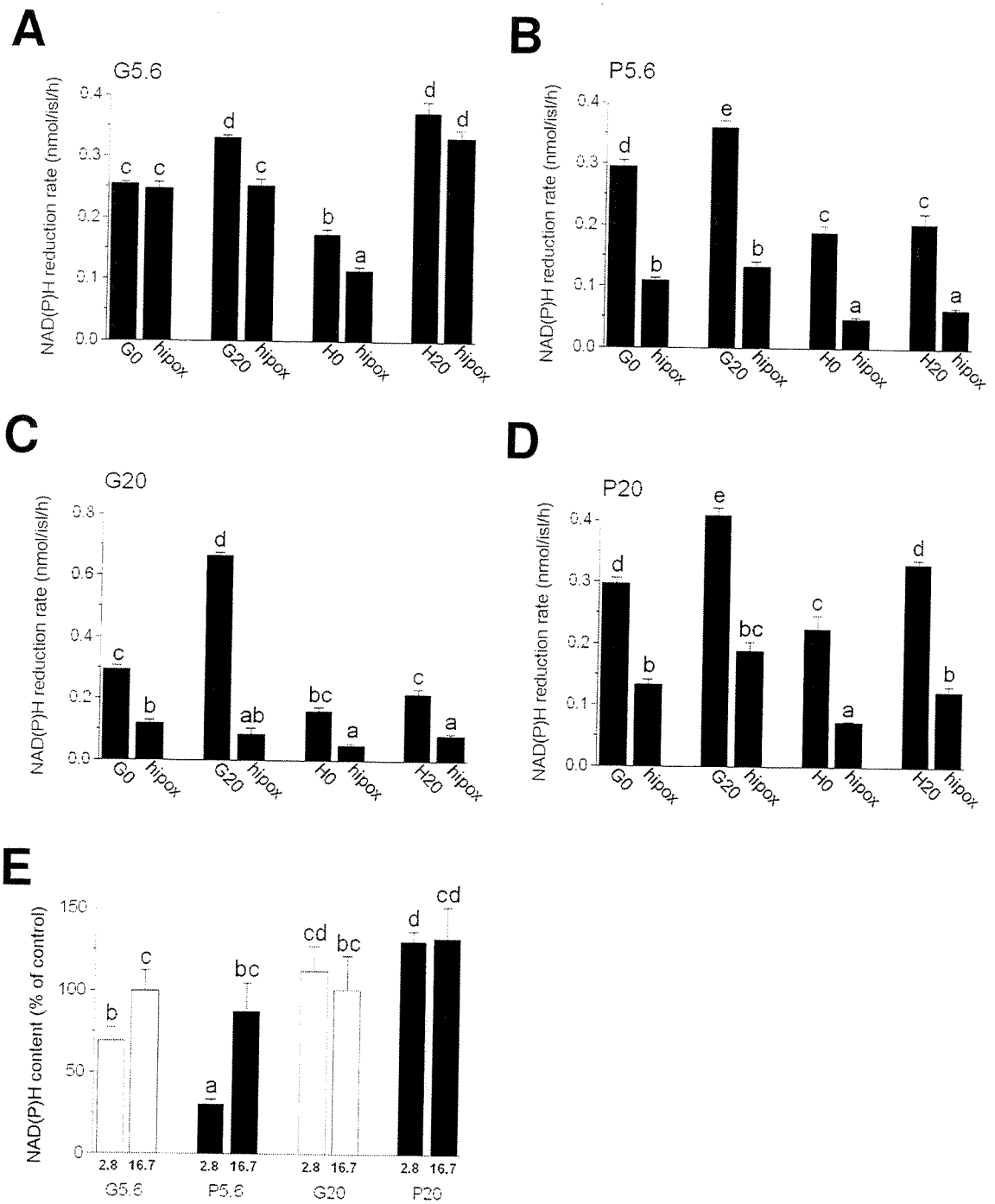
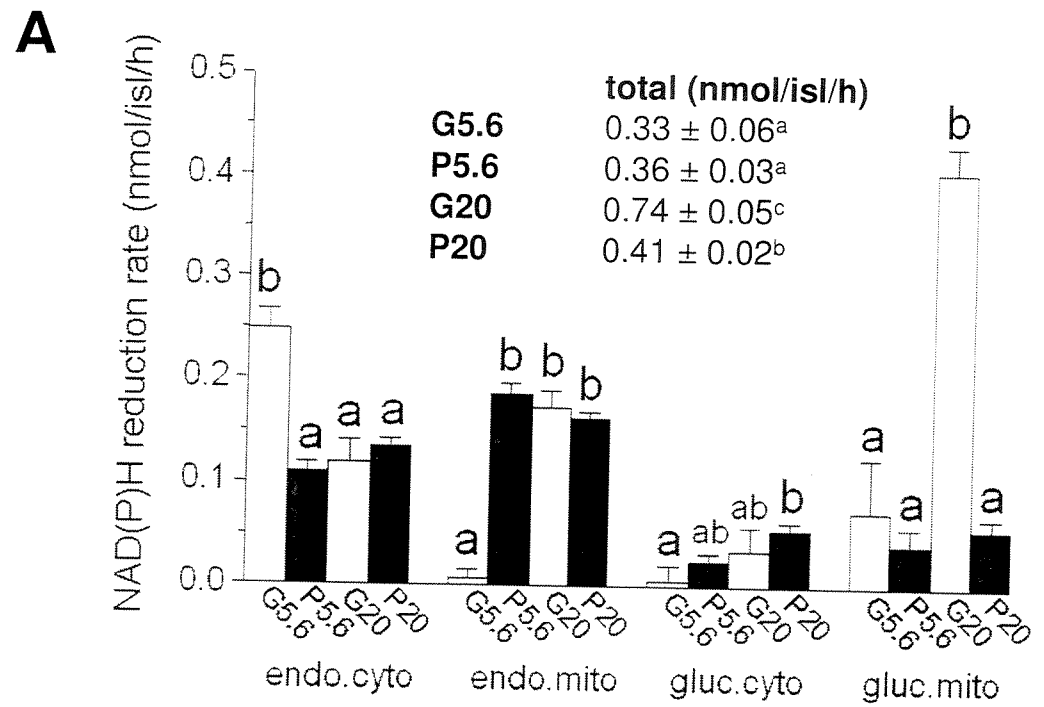
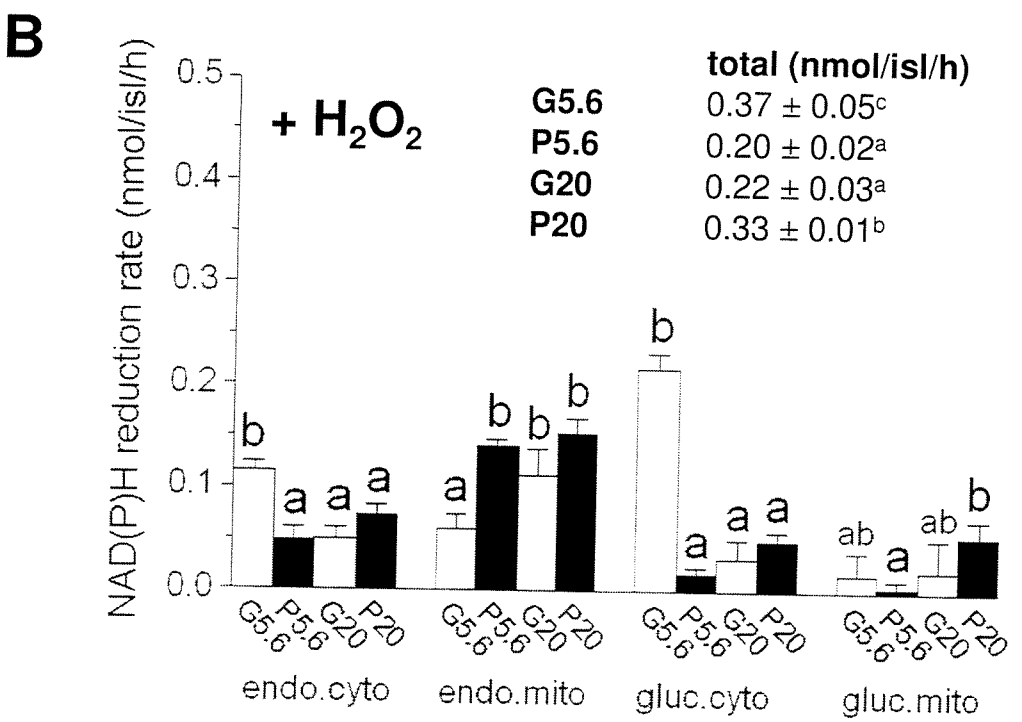

A
Glucose Oxidation (% of control)
150
100
50
0
a
a
c
b
b
ab
d
d
G5.6/2.8
G5.6/16.7
P5.6/2.8
P5.6/16.7
G20/2.8
G20/16.7
P20/2.8
P20/16.7
B
Insulin Secretion (% of control)
100
75
50
25
0
bc
a
b
a
c
bc
c
b
G5.6/2.8
G5.6/16.7
P5.6/2.8
P5.6/16.7
G20/2.8
G20/16.7
P20/2.8
P20/16.7

Figure 5


G5.6
P5.6
G20
P20
GSIS (fold increase)
Rotenone (nM)
a
a
b
c
0
1
2
3
4
0
20
40
60
80
100


A
G5.6
NAD(P)H reduction rate (nmol/isl/h)
0.0
0.1
0.2
0.3
0.4
c c d c b a d d
G0 hipox G20 hipox H0 hipox H20 hipox
B
P5.6
NAD(P)H reduction rate (nmol/isl/h)
0.0
0.1
0.2
0.3
0.4
d b e b c a c a
G0 hipox G20 hipox H0 hipox H20 hipox
C
G20
NAD(P)H reduction rate (nmol/isl/h)
0.0
0.2
0.4
0.6
0.8
c b d ab bc a c a
G0 hipox G20 hipox H0 hipox H20 hipox
D
P20
NAD(P)H reduction rate (nmol/isl/h)
0.0
0.1
0.2
0.3
0.4
d b e bc c a d b
G0 hipox G20 hipox H0 hipox H20 hipox
E
NAD(P)H content (% of control)
0
50
100
150
b c a bc cd bc d cd
2.8 16.7 2.8 16.7 2.8 16.7 2.8 16.7
G5.6 P5.6 G20 P20


A
NAD(P)H reduction rate (nmol/isl/h)
0.5
0.4
0.3
0.2
0.1
0.0
total (nmol/isl/h)
G5.6 0.33 ± 0.06a
P5.6 0.36 ± 0.03a
G20 0.74 ± 0.05c
P20 0.41 ± 0.02b
G5.6 P5.6 G20 P20
endo.cyto
endo.mito
gluc.cyto
gluc.mito


B
+ H2O2
NAD(P)H reduction rate (nmol/isl/h)
0.5
0.4
0.3
0.2
0.1
0.0
total (nmol/isl/h)
G5.6 0.37 ± 0.05c
P5.6 0.20 ± 0.02a
G20 0.22 ± 0.03a
P20 0.33 ± 0.01b
G5.6 P5.6 G20 P20
endo.cyto
endo.mito
gluc.cyto
gluc.mito

Conclusão

O sistema varredor de EROs na ilhota de Langerhans é composto pelas isoformas da SOD, que converte O_2^- em H_2O_2 (35); tiol-redutases (36), que regeneram glutationa e tioredoxina a partir de NADPH mitocondrial (34); e a calatase, que utiliza NADPH em baixas concentrações de H_2O_2 (16). Um dos trabalhos apresentados aqui está entre os primeiros a mostrar atividade da catalase em concentrações fisiológicas de H_2O_2 (2^o trabalho). Em outro, confirmamos a presença do sistema tioredoxina/tioredoxina peroxidase em células β, o qual também utiliza NADPH como fonte de elétrons (37). Dessa forma, parece que boa parte do sistema varredor da ilhota está ligada a sua capacidade de gerar NAD(P)H.

Vários trabalhos associam a ativação de genes pró-apoptóticos com altas concentrações de glicose em ilhotas de ratos adultos, mantidas em cultura (38). Entretanto, encontramos pouca evidência de efeito tóxico da glicose sobre as ilhotas de neonatos, não havendo perda da GSIS após 4d em cultura com 22.4 mM de glicose (1^o trabalho). Da mesma forma, não observamos efeito tóxico do NO gerado em resposta ao estresse causado por H_2O_2, uma vez que o aumento da produção deste radical não foi acompanhado por lesão das ilhotas. Na época da publicação do 1^o trabalho, havia um grande esforço em se ligar à produção de NO e a apoptose em células β, como se o radical em si exercesse ação nociva. No entanto, a concentração intracelular de NO é baixa e alguns trabalhos importantes questionaram se o NO não seria apenas um indicativo de apoptose (39). Neste 1^o trabalho, então, não pudemos descartar a iNOS como marcador de dano celular, mas entendemos que, se sua expressão é de fato danosa à célula β, esse dano se deva ao elevado consumo de NAD(P)H pela enzima, podendo provocar depleção do nucleotídeo (40).

Em processos inflamatórios agudos, as ilhotas podem ser expostas a concentrações locais de 1 mM de H_2O_2 (41). Iniciamos então os experimentos com populações heterogêneas de ilhotas, as quais foram selecionadas em 4d de cultura por sua resistência a 1 mM de H_2O_2. Nessas condições, quando quase toda a atividade peroxidase nas ilhotas se deve a catalase, os grupos de ilhotas resistentes ao peróxido mostraram maior atividade dessa enzima. Porém, somente na presença de fatores anti-apoptóticos (42) ou 22.4 mM de glicose as ilhotas passaram pela cultura com H_2O_2 sem sofrer dano. Uma vez que a leucina ativa o metabolismo mitocondrial das células β e não ofereceu proteção contra o H_2O_2, deduzimos que o sistema antioxidante das células secretoras de insulina esteja mais vinculado ao metabolismo citoplasmático.

Embora, certamente, leve à perda da GSIS, *in vivo* o estresse agudo, exemplificado acima, não é a forma mais comum de lesão sobre as ilhotas. Exceto em situações patológicas de inflamação e infiltração das ilhotas por linfócitos, o H_2O_2 na ilhota somente atinge concentrações de alguns poucos micromolares (10). No segundo trabalho, adicionando-se 5 μM de H_2O_2 a diferentes extratos de ilhotas, observamos que a catalase só possui atividade peroxidase detectável em concentrações de enzima muito superiores ao medido nesse tecido, mesmo na presença de NADPH (2^o trabalho). Na literatura, é descrita uma proteção, apenas parcial, da catalase contra o dano à secreção de insulina, mediado por H_2O_2 (13). Ainda, em presença de 5 μM de H_2O_2, amostras de tecidos diferentes com atividade de catalase variando entre 3% e 1200% do encontrado nas ilhotas mostraram atividades peroxidase semelhantes. Entretanto, mesmo nessa concentração de peróxido todos os tióis usados mostraram alta reatividade com o H_2O_2. Verificamos então que proteínas da ilhota, do plasma ou mesmo albumina adquirem atividade peroxidase na presença de tióis ou NADPH. Isso sugere que muitas proteínas na célula β atuam como peroxidases em condições fisiológicas, se a célula mantiver uma produção suficiente de NADPH (43).

Para localizar e compreender a química da atividade peroxidase que ocorre em baixas concentrações de H_2O_2, separamos por centrifugação os homogenatos de ilhotas em duas frações: uma hidrossolúvel e outra contendo núcleos e organelas. Ambas as frações mostraram considerável atividade peroxidase, mas apenas a fração de organelas se mostrou sensível a NADPH e tióis. Por outro lado, na fração hidrossolúvel a atividade redutora do NADPH foi inibida por um quelante de Fe^{3+}/Cu^{2+}, sugerindo que a degradação do H_2O_2 ocorra na interação com NADPH e íons na solução, como nas reações de Fenton (6). Segundo dados apresentados no 2^o trabalho, tióis dissolvidos podem reduzir íons presentes na solução e estes transferirem elétrons aos tióis de proteínas, em especial da fração de organelas. Como os tióis celulares são mais reativos ao H_2O_2 que as peroxidases, a falta de NAD(P)H levaria rapidamente a sua inativação, com alteração de toda atividade enzimática na célula β que esteja ligada ao estado redox das enzimas (4, 27). Portanto, é provável que a principal proteção da célula β contra os EROs esteja em proteínas que servem de alvo preferencial para ataques químicos, mas, que são regeneradas por NADPH ou tióis em solução. Localizada nas frações nuclear e citossólica dos homogenatos, a catalase teria atividade significativa somente onde estivesse em altas concentrações ou, em situações de estresse oxidativo bastante aumentado, o que levaria a requisição de quantidades adicionais de NADPH proveniente do metabolismo celular (44).

Não observamos diferenças entre a GSIS de ilhotas cultivadas com menos de 200 μM de H_2O_2, comparado ao controle. Assim, buscamos concentrações ideais de H_2O_2 semelhantes às encontradas em situações de estresse *in vivo*, o que foi obtido cultivando as ilhotas por 4d em meio contendo 200 μM de H_2O_2 por 4d. Nessas condições, as ilhotas resistentes foram selecionadas. Em experimentos onde os extratos dessas ilhotas resistentes eram expostos a 5 μM de H_2O_2, não detectamos alteração de atividade peroxidase, mostrando que a resistência das ilhotas ao H_2O_2 não está relacionada com expressão das peroxidases de baixo K_m. A mesma requer a atividade da catalase e um suprimento adicional de glicose (1º trabalho). É provável que a glicose protege as ilhotas pela geração de NADPH, pois observamos que tanto ilhotas selecionadas por cultura com H_2O_2 como as cultivadas em 20 mM de glicose mostravam elevado uso da glicose na via das pentoses (3º trabalho).

Outras alterações comuns nas ilhotas selecionadas por H_2O_2 ou por altas concentrações de glicose foram o aumento de expressão da enzima glicose-6-fosfato dehidrogenase (via das pentoses) e metabolismo mitocondrial aumentado. Sabe-se que a via das pentoses é uma defesa antioxidante de células embrionárias (20). Nas ilhotas de adultos, menos de 5% da glicose é destinado à via das pentoses, chegando a 40% nas ilhotas de neonatos (3º trabalho, 45). Nas ilhotas imaturas de ratos neonatos, a produção mitocondrial de NAD(P)H é muito baixa (45). Nessas ilhotas, observamos prevalência da produção de NAD(P)H a partir de substratos endógenos (ácidos graxos, aminoácido, etc), ao invés da glicose. Apenas nas ilhotas selecionadas por cultura com H_2O_2 ou por altas concentrações de glicose, entretanto, essa produção de NAD(P)H é um processo aeróbico.

A ativação da via das pentoses em ilhotas cultivadas com 20 mM de glicose sugere que a glicose *per se* produz estresse oxidativo, a chamada glicotoxicidade. Segundo a literatura, o metabolismo aumentado de glicose ou ácido graxos, de alguma forma, ativa proteínas como NF-kB e PKC-δ, iniciadores do processo apoptótico na célula β (46)**.** De fato, ilhotas cultivadas com H_2O_2 ou altas concentrações de glicose mostraram maior resposta à estimulação das PKCs -δ, -ε ou -μ por phorbol-miristato (dados ainda não publicados). No entanto, a incubação com 200 μM de H_2O_2 inibiu a geração de NAD(P)H mitocondrial em resposta à glicose. Isso sugere que os efeitos da glicose e do H_2O_2 sobre as ilhotas podem ser semelhantes, mas as semelhanças parecem ser limitadas à ativação da via das pentoses.

Além disso, as ilhotas cultivadas com altas concentrações de glicose aumentaram o uso da lançadeira de NADH glicerol-fosfato, enquanto o H_2O_2 selecionou ilhotas com maior uso da lançadeira de malato/aspartato (47). Embora nenhuma delas aumente a oxidação de glicose nas ilhotas de neonatos, o sistema de lançadeiras mostrou-se fundamental à GSIS. Isso reflete o transporte reduzido de carbonos entre o citossol e as mitocôndrias nas ilhotas de ratos neonatos, onde é mesmo possível que muito do NAD(P)H usado na síntese de ATP provenha de NAD(P)H citoplasmático transportado pelas lançadeiras.

Finalmente, sendo o NAD(P)H citoplasmático um substrato necessário tanto a GSIS quanto ao sistema varredor da ilhota, concluímos que sua depleção por estresse oxidativo provoca, ao mesmo tempo, dano celular e perda da GSIS. Devido à baixa atividade mitocondrial das ilhotas de neonatos, a sobrevivência dessas ilhotas expostas ao H_2O_2 e sua capacidade de secretar insulina em resposta à glicose parecem ambas determinadas pela geração citossólica de NAD(P)H e o transporte desse para a matriz mitocondrial, através do sistema de lançadeiras (10).

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