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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 $NADP^{\cdot}$ and $RS^{\cdot}$ 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^- 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 [1]. 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^- 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 $NADP^+ + O_2^-$) 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.

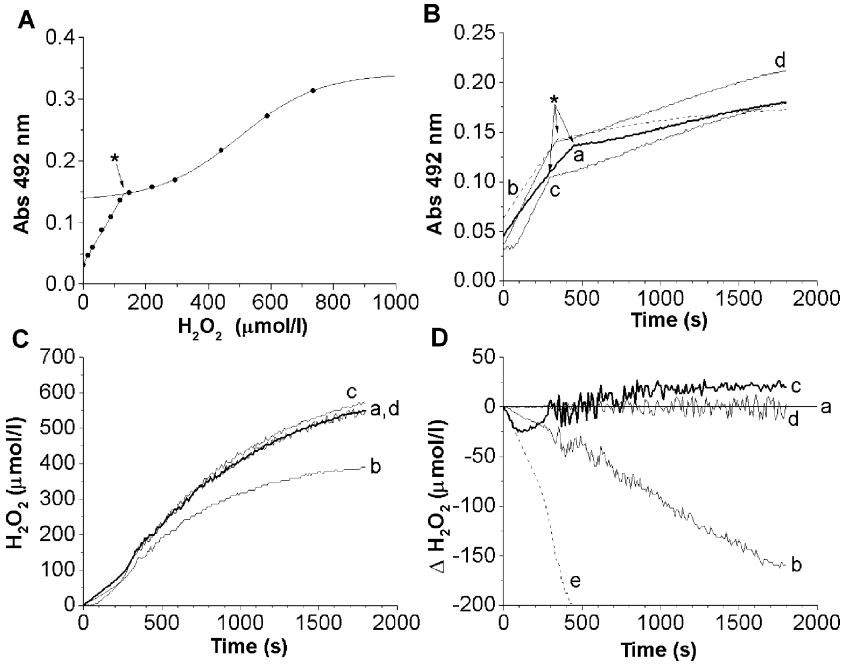

Fig. 1. Time course of H_2O_2 detection. (A) Standard curve constructed with known concentrations of H_2O_2. (B) Typical curves of H_2O_2 detection from samples containing (a) Hanks pH 7.4, (b) soluble islet extract 200 μg/ml of protein, (c) L-cysteine 50 μmol/l, and (d) cystine 25 μmol/l. (C) Detected 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

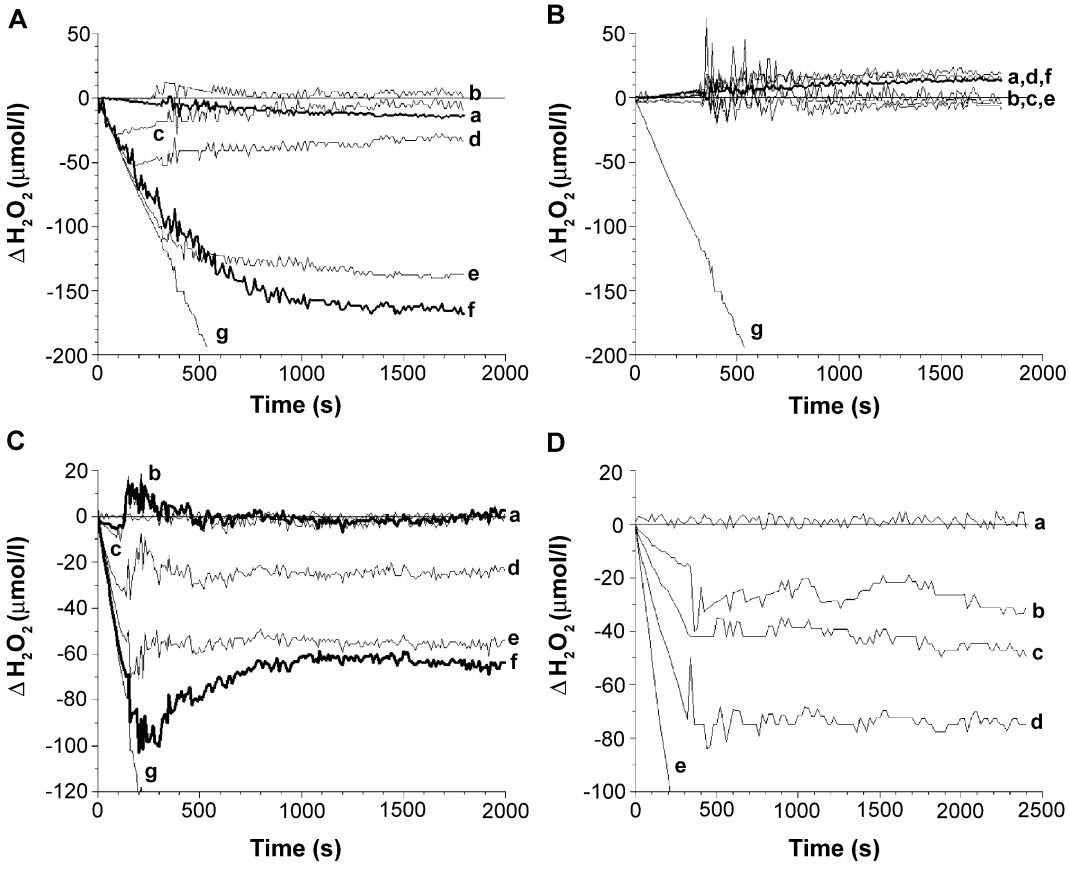

Fig. 2. Determination of the reactivity of small molecules with H_2O_2. Each reagent has been added prior to the beginning of the reaction. (A) L-cysteine was added at concentrations of (a) 0, (b) 35, (c) 70, (d) 100, (e) 140, and (f) 180 μmol/l. (B) Cystine was added at concentrations of (a) 0, (b) 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).

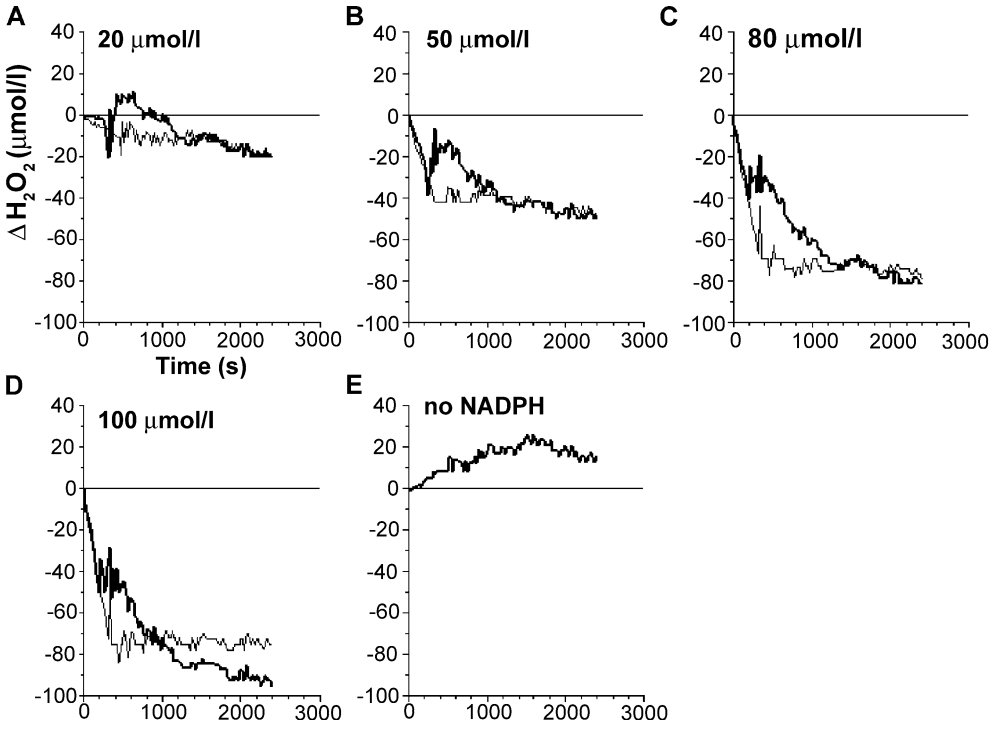

Fig. 3. Catalase activity with addition of NADPH. NADPH was added to the media without (thin lines) or with 26 μg/ml catalase (shaded lines). The concentrations of NADPH are indicated. Each line is representative of three independent experiments and shows the difference between detected H_2O_2 in samples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

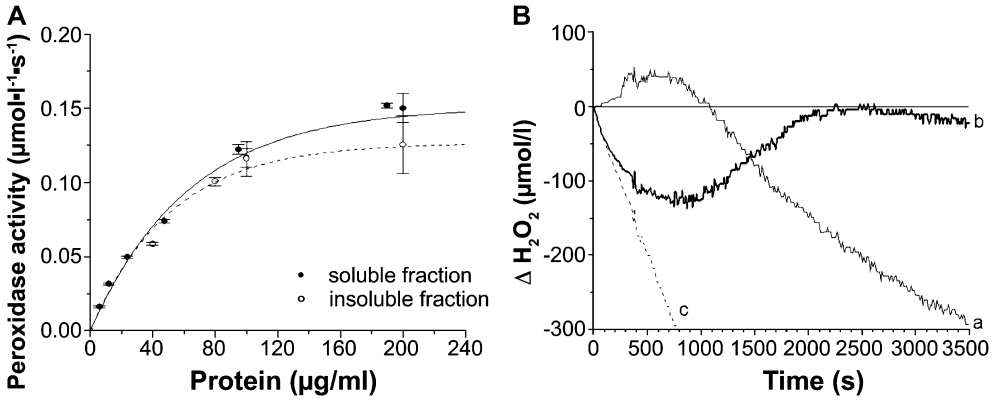

Fig. 4. Intrinsic peroxidase activity of islet extracts. (A) Activities were measured as maximal H_2O_2 degradation rate compared to blanks (Hanks alone). Each point is mean ± S.E. of four experiments. (B) Typical activity of denatured soluble extract. Samples were heated for 5 min at 100 °C and cooled 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 1 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 1 conversion to compound 2, yielding $NADP^{\cdot}$ and thus O_2^{-}, its product by reaction with molecular oxygen [15,21]. NADPH competes with 4AA for compound 1 decreasing the detected amount of 492 nm colored quinone. The generated O_2^{-} is an inhibitor of HRP activity.

Proteins able to trap O_2^{-} (like the SOD isoforma) 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]. Fig. 3E 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 1 (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 1 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

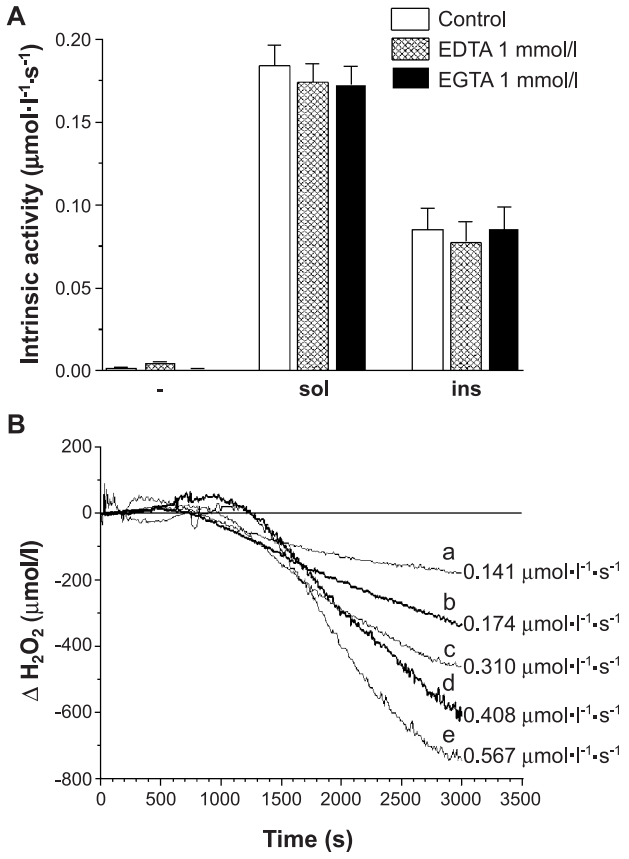

Fig. 5. Intrinsic peroxidase activities of samples exposed to chelating agents. Hanks solution, soluble (sol) or insoluble (ins) islet extracts with (A) EDTA or EGTA; (B) cyanide at concentrations of (a) 0, (b) 2, (c) 4, (d) 6, and (e) 10 mmol/l. Each line is representative of four independent experiments.

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 [1]. 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 $\mu mol \cdot l^{-1} 1 \cdot s^{-1}$ 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 $RS^{\bullet}$ 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 $CS^{\bullet}$ 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.

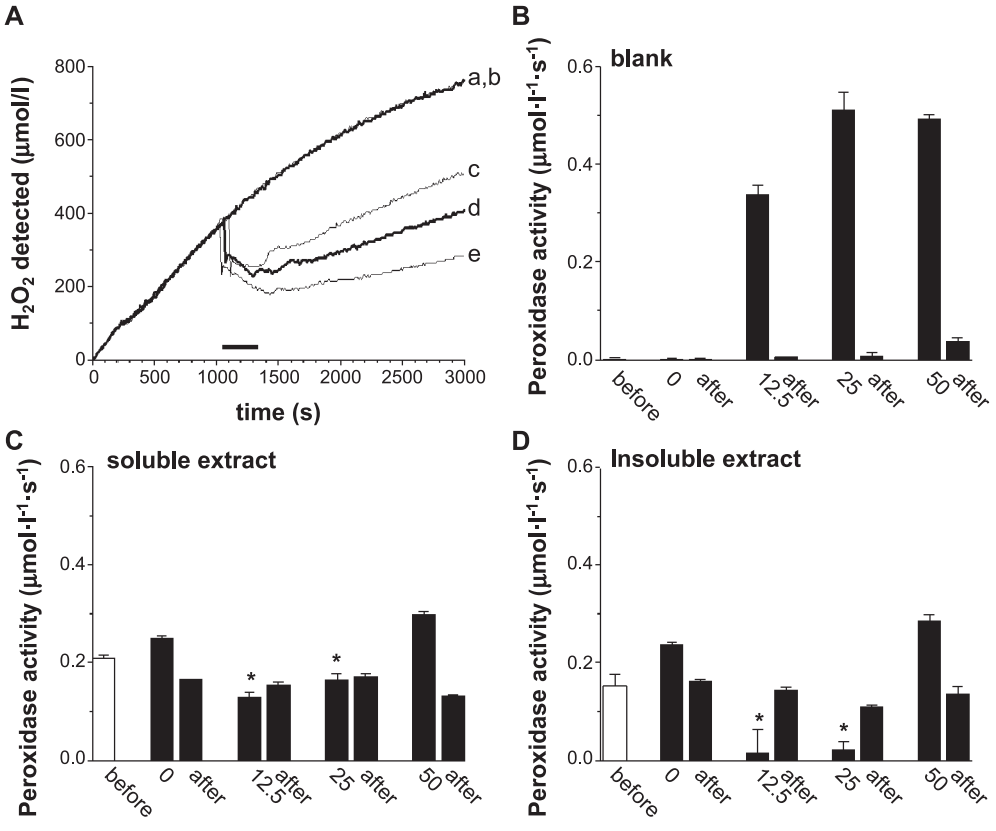

Fig. 6. Peroxidase activities of samples affected by thiol addition. (A) Typical H_2O_2 detection curves with 10 μl of L-cysteine added at $t = 1000$ s to final concentrations of (b) 0, (c) 12.5, (d) 25, and (e) 50 μmol/l. In line (a), there was no addition. (B) Peroxidase activities attributed to the 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.

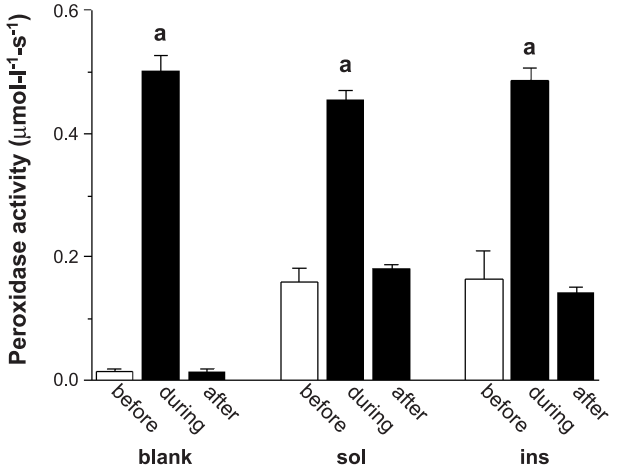

Fig. 7. Protective effect of cyanide on thiol-induced inhibition of peroxidase activity. Islet extracts were incubated for 30 min at 37 °C with at least 5 mmol/l of KCN and adjusted to final concentrations of 100 μg/ml of total protein and 1 mmol/l cyanide. Cysteine (50 μmol/l) was added at $t = 1000$ 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].

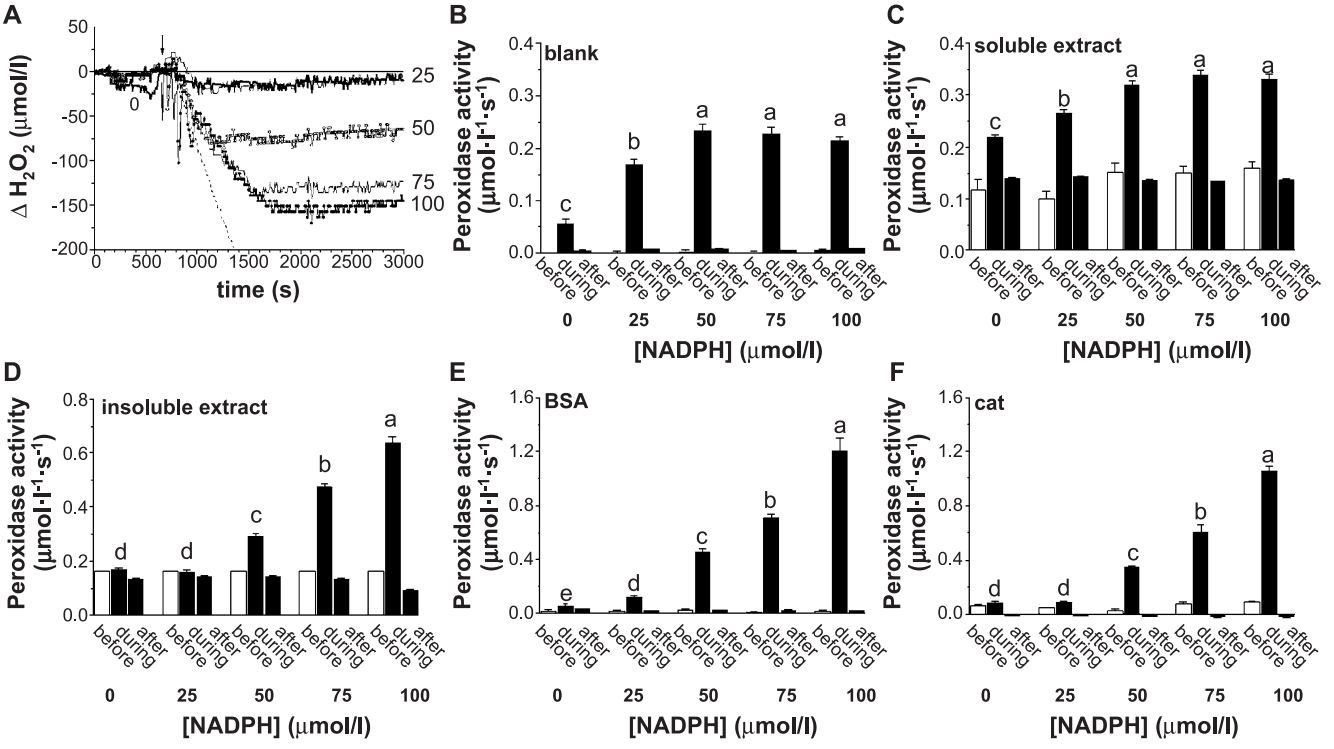

Fig. 8. Effect of NADPH addition on peroxidase activity. (A) Typical curves representing the differences between detected H_2O_2 in samples and in the blank (no NADPH). Concentrations of NADPH (arrow) in μmol/l are indicated. The dashed line represents the expected result if no H_2O_2 was detected. (B), (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 $NADP^{\cdot}$ 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 $NADP^{\bullet}$ 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 $NADP^{\bullet}$ production as the reason NADPH/protein decreases (data not shown).

O-phenantroline per se did not alter the $NADP^{\cdot}$ 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 $NADP^{\bullet}$ generation by any of the samples (data not shown).

Treatment of the samples with 1 mmol/l cyanide caused no inhibition of $NADP^{\cdot}$ 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 $NADP^{\cdot}$ production by HRP compound 1 (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 1. In a H_2O_2-rich medium, catalase compound 1 is readily re-converted to ferricatalase through a two-electron reaction with H_2O_2. However, in a H_2O_2-poor

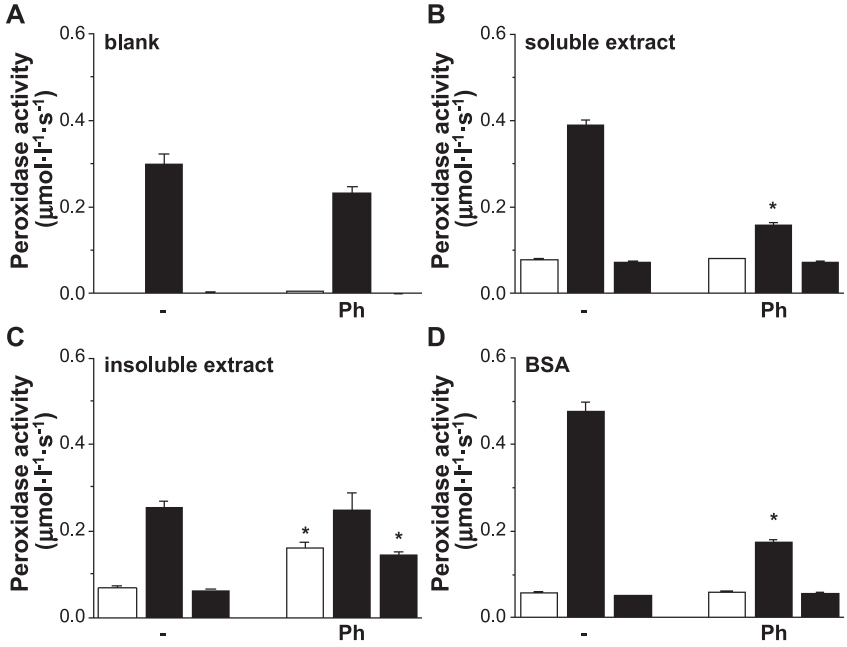

Fig. 9. Effect of *o*-phenantroline on $NADP^{\cdot}$ production. NADPH (50 μmol/l) was added at $t = 600$ s. Bars are mean ± S.E. of four experiments and represent the H_2O_2 disappearance due to $NADP^{\cdot}$ production before (white bars), during, and after NADPH consumption. In panel A, no protein 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 $NADP^{\cdot}$ production, indicating that BSA per se is able to chelate or oxidize NADPH.

If metal ions participate in some O_2^- 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^- 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 ($RSO^{\cdot}$), 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:

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

$$\text{CS}^{\bullet} + \text{H}_2\text{O}_2 \Rightarrow \text{CSO}^{\bullet} + \text{H}_2\text{O} \quad (2\text{A})$$

$$\text{CSO}^{\bullet} + \text{H}^{+} + \text{CSH} \Rightarrow \text{CSSC} + \text{H}_2\text{O} \quad (2\text{B})$$

$$\text{ins}[\text{metal}^{2+}](\text{inactive}) + \text{O}_2 \Rightarrow \text{ins}[\text{metal}^{3+}](\text{active}) + \text{O}_2^- \quad (3)$$

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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References

[1] L.F. Stoppiglia, T.A. Nogueira, A.R. Leite, E.M. Carneiro, A.C. Boschero, Biochim. Biophys. Acta 1588 (2002) 113–118.
[2] M.J. MacDonald, Arch. Biochem. Biophys. 305 (1993) 205–214.
[3] Y. Tsuura, H. Ishida, T. Shinomura, M. Nishimura, Y. Seino, Biochem. Biophys. Res. Commun. 252 (1998) 34–38.
[4] D.J. Pinero, J. Hu, B.M. Cook, R.C. Scaduto Jr., J.R. Connor, Biochim. Biophys. Acta 1497 (2000) 279–288.
[5] M.L. Lang, M.A. Kerr, Biochem. Biophys. Res. Commun. 276 (2000) 749–755.
[6] M. Tiedge, S. Lortz, J. Drinkgern, S. Lenzen, Diabetes 46 (1997) 1733–1742.
[7] L.E.S. Netto, H.Z. Chae, S.W. Kang, S.G. Rhee, E.R. Stadtman, J. Biol. Chem. 271 (1996) 15315–15321.
[8] A. Salvador, J. Souza, R. Pinto, Free Radic. Biol. Med. 10 (2001) 1208–1215.
[9] D.L. Eizirik, M. Flodstrom, A.E. Karlsen, N. Welsh, Diabetologia 39 (1996) 875–890.
[10] E. Neyens, J. Baeyens, J. Hazard Mater. 98 (2003) 33–50.
[11] K. Echtay, D. Roussel, J. St-Pierre, M.B. Jekabson, S. Cadenas, J.A. Stuart, J.A. Harper, S.J. Roebuck, A. Morrison, S. Pickering, J.C. Claphan, M. Brand, Nature 415 (2002) 96–99.
[12] K. Sakai, K. Matsumoto, T. Nishikawa, M. Suefuji, K. Nakamaru, Y. Hirashima, J. Kawashima, T. Shirotani, K. Ichinose, M. Brownlee, E. Araki, Biochim. Biophys. Res. Commun. 1 (2003) 216–222.
[13] G.H. Patterson, S.M. Knobel, P. Arkhammar, O. Thastrup, D.W. Piston, Proc. Natl. Acad. Sci. 97 2000, pp. 5203–5207.
[14] H.N. Kirkman, M. Rolfo, A.M. Ferraris, G.F. Gaetani, J. Biol. Chem. 274 (1999) 13908–13914.
[15] V. De Sandro, C. Dupuy, J. Kaniewski, R. Ohayon, D. Dème, A. Virion, J. Pommier, Eur. J. Biochem. 201 (1991) 507–513.
[16] K.I. Minard, L. McAlister-Henn, Free Radic. Biol. Med. 31 (2001) 832–843.
[17] S. Filosa, A. Fico, F. Paglialunga, M. Balestrieri, A. Crooke, P. Verde, P. Abrescia, J.M. Bautista, G. Martini, Biochem. J. 370 (2003) 935–943.
[18] K. Anjaneyulu, R. Anjaneyulu, A. Sener, W.J. Malaisse, Biochimie 64 (1982) 29–36.
[19] E.C. Vieira, E.M. Carneiro, M.Q. Latorraca, V. Delguingaro-Augusto, M.E.C. Amaral, J.R. Bosqueiro, A.C. Boschero, J. Nutr. Biochem 12 (2001) 285–291.
[20] C.C. Winterbourn, D. Metodiewa, Free Radic. Biol. Med. 27 (1999) 322–328.
[21] J.V. Steveninck, J.P.J. Boegheim, T.M.A.R. Dubbelman, J.V.D. Zee, Biochem. J. 242 (1987) 611–613.
[22] J.L. Hirpara, M.V. Clément, S. Pervaiz, J. Biol. Chem. 1 (2001) 514–521.
[23] U. Burner, C. Obinger, FEBS Lett. 411 (1997) 269–274.
[24] C.D. Putnam, A.S. Arvai, Y. Bourne, J.A. Tainer, J. Mol. Biol. 296 (2000) 295–309.
[25] M. Akagawa, K. Suyama, Free Radic. Res. 36 (2002) 13–21.
[26] P.J. Hart, M.M. Balbirnie, N.L. Ogihara, A.M. Nersissian, M.S. Weiss, J.S. Valentine, D. Eisenberg, Biochemistry 38 (1999) 2167–2178.
[27] G.E.O. Borgstahl, M. Pokross, R. Chehab, A. Sekher, E.H. Snell, J. Mol. Biol. 296 (2000) 951–959.
[28] M. Fournier, Y. Zhang, J.D. Wildschut, A. Dolla, J.K. Voordouw, D.C. Schriemer, G. Voordouw, J. Bacteriol. 185 (2003) 71–79.
[29] D. Mercan, M.M. Kadiata, W.J. Malaisse, Biochem. Biophys. Res. Commun. 262 (1999) 346–349.
[30] H.R. Oliveira, R. Verlengia, C.R. Carvalho, L.R. Britto, R. Curi, A.R. Carpinelli, Diabetes 52 (2003) 1457–1463.
[31] M.K. Cha, I.H. Kim, Biochem. Biophys. Res. Commun. 222 (1996) 619–625.
[32] S. Carballal, R. Radi, M.C. Kirk, S. Barnes, B.A. Freeman, B. Alvarez, Biochemistry 42 (2003) 9906–9914.
[33] H. Zhang, U.T. Brunk, Diabetologia 36 (1993) 707–715.
[34] S. Rasilainen, J.M. Nieminen, A.L. Levonen, T. Otonkoski, R. Lapatto, Biochem. Pharmacol. 63 (2002) 1297–1304.