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Recycling Processes of Cellular Ascorbate Generate Oxidative Stress in Pancreatic Tissues in In Vitro System

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Ascorbate is a reducing agent, which is also known to oxidize cellular components. Our proposed mechanism of the oxidative action is as follows: Ascorbate is concentrated in the pancreas and is leaked in adverse conditions, and oxidized to dehydroascorbate. The dehydroascorbate is carried into cells by a glucose transporter (GLUT) and reduced back to ascorbate. The reduction processes take electrons from other cellular components. Ascorbate or dehydroascorbate treatment elevated thiobarbituric acid–reactive substance (TBARS) concentrations in pancreas. The elevations in TBARS concentrations were blocked by cytochalasin B, a GLUT inhibitor. To confirm further the prooxidative action, changes in glutathione content were quantified. Glutathione concentrations were lower in ascorbate- or dehydroascorbate-treated groups. The ascorbate-induced decrease in glutathione was blocked by cytochalasin B. To prevent oxidation of ascorbate to dehydroascorbate, glutathione was added to the medium. The ascorbate plus glutathione and dehydroascorbate plus glutathione groups showed lower TBARS concentrations than those of the ascorbate and dehydroascorbate groups, respectively. There were changes in the morphology of Langerhans islets following ascorbate treatment, which disappeared following treatment with ascorbate plus cyto-chalasin B. The observations indicate that ascorbate generates oxidative stress and affects the structure of islets.

Key Words: Thiobarbituric acid–reactive substance; glutathione; glucose transporter; cytochalasin B; Langerhans islet.

Introduction

Ascorbate is a well-known reducing agent. However, it is also known to oxidize cellular components in certain experimental conditions. We have attempted to clarify the mechanism of ascorbate-induced oxidative stress for some time *(1–3)*. Our proposed mechanism is as follows: Ascorbate is highly concentrated in several organs in the body such as the adrenal glands, brain, ovaries, and pancreas *(4,5)*. The high concentrations of ascorbate cannot be sustained without adequate supply of nutrients and oxygen but are lost simply by leaking out of the cells or by reverse transportation *(6)*. The ascorbate that is leaked out of cells is oxidized to dehydroascorbate by molecular oxygen in extracellular fluid. The dehydroascorbate is carried into cells by a glucose transporter (GLUT) *(7–9)*. The oxidized product of ascorbate (dehydroascorbate) is reduced by both enzymatic *(10–12)* and nonenzymatic processes. The reduction processes to ascorbate take electrons from other cellular components, and thus the cellular molecules that have lost electrons during the reduction processes are oxidized, generating oxidative stress. If vital components in cells are oxidized, the oxidation triggers cascade reactions of apoptosis.

Although the pancreas contains a high concentration of ascorbate *(4)*, the physiologic significance of ascorbate in the pancreas has not been closely examined. In the present study, we observed that ascorbate generated oxidative stress in rat pancreas. The pancreas consists of a heterogeneous cell population, mainly composed of acinar cells, and endocrine cells in Langerhans islets. Ideally, we should isolate the islets and examine the effect of ascorbate on them. However, it is difficult to harvest large numbers of islets. We have therefore examined morphologic changes in Langerhans islets in pancreatic tissues to confirm whether they were involved in the oxidative stress during ascorbate treatment. We anticipate that our observations will help to clarify the physiologic and pathologic roles of pancreatic ascorbate.

Results

Ascorbate (3 m*M*) treatment elevated thiobarbituric acid–reactive substance (TBARS) concentration from 100 ± 15% (control) to 174 ± 27% ($p < 0.05$) (Fig. 1A), and dehydroascorbate (3 m*M*) also elevated TBARS concentration from 100 ± 15% (control) to 272 ± 44% ($p < 0.01$) (Fig. 1B) after a 3-h incubation. We chose a 3-h incubation period based on our previous studies *(2,3)*. The dose-response relationships between ascorbate and TBARS (Fig. 1A), and dehydroascorbate and TBARS (Fig. 1B) indicated that potency of dehydroascorbate on TBARS production was higher than that of ascorbate.

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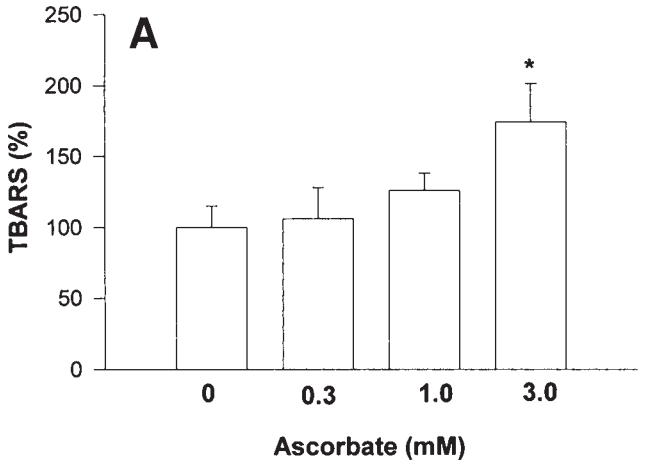


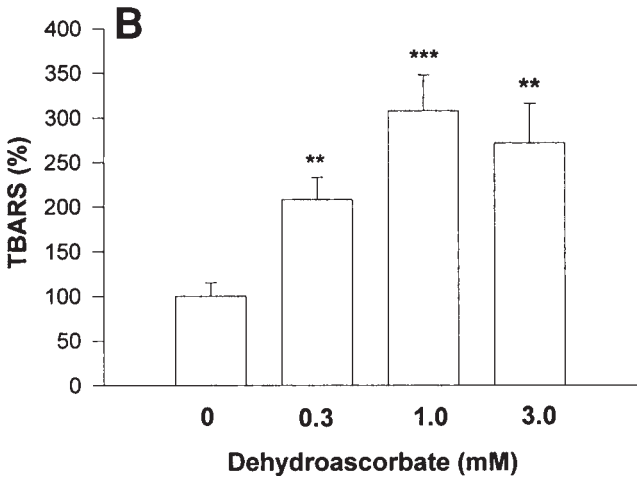
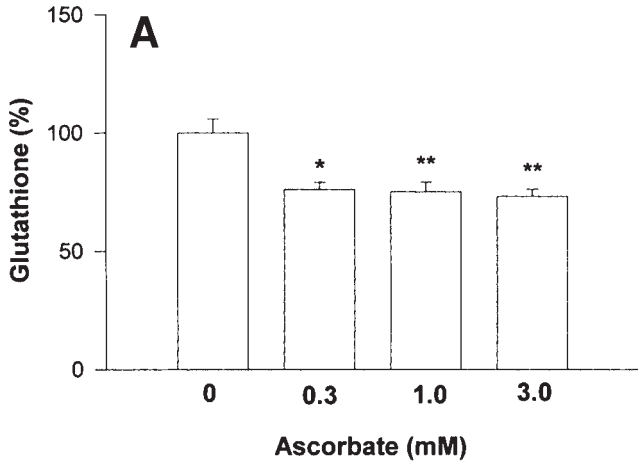


Fig. 1. Effects of (**A**) ascorbate and (**B**) dehydroascorbate on TBARS production. Quantities are expressed in percentages of control values. Each vertical bar represents the mean ± SEM. Six independent experiments were performed. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.



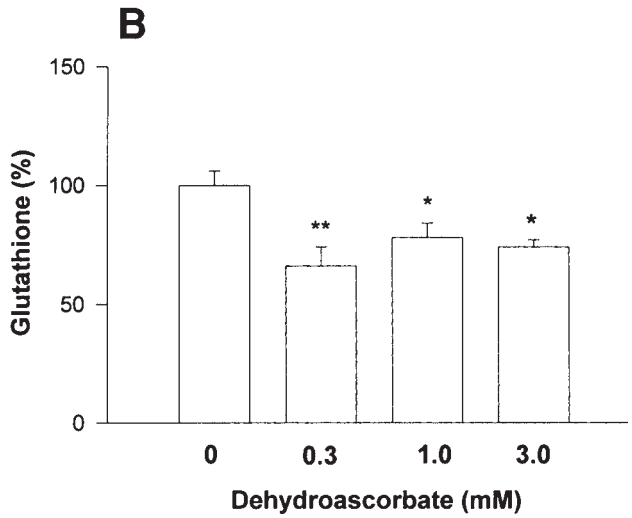


Fig. 2. Effects of (**A**) ascorbate and (**B**) dehydroascorbate on glutathione concentrations. Quantities are expressed in percentages of control values. Each vertical bar represents the mean ± SEM. Six independent experiments were performed. *$p < 0.05$; **$p < 0.01$.

To confirm further the prooxidative action of ascorbate and dehydroascorbate, we quantified changes of glutathione contents in the pancreatic tissues. Concentrations of glutathione were lower (75 ± 3%; $p < 0.01$) in the ascorbate (1.0 m*M*)-treated group than the control group (100 ± 6%). Similarly, the 3.0 m*M* ascorbate-treated group showed lower concentrations of glutathione (73 ± 3%; $p < 0.01$) than the control group (Fig. 2A). Samples treated with 1.0 m*M* dehydroascorbate decreased glutathione concentrations (78 ± 6%; $p < 0.05$), and a higher concentration of dehydroascorbate (3.0 m*M*) also decreased glutathione concentration (74 ± 3%; $p < 0.05$ (Fig. 2B).

When we measured the glutathione content in pancreatic tissue after treatment with saline (control), ascorbate (3 m*M*), or ascorbate (3 m*M*) plus cytochalasin B (0.3 m*M*), the glutathione concentration was decreased in the ascorbate-treated group (72 ± 3; $p < 0.01$), but the ascorbate-induced decrease in glutathione concentration was blocked by cytochalasin B (90 ± 2%; $p < 0.05$ for ascorbate vs ascorbate plus cytochalasin B) (Fig. 3). When samples were treated with control, dehydroascorbate, or dehydroascorbate plus cytochalasin B, we observed similar results as for previous experiments with ascorbate (74 ± 2% for dehydroascorbate, 109 ± 6 for dehydroascorbate plus cytochalasin B; $p < 0.05$) (Fig. 3).

To prevent oxidation of ascorbate to dehydroascorbate in medium during an incubation period, 3 m*M* glutathione was added to the medium. The glutathione did not change the TBARS concentration. The glutathione-treated group (3 m*M* ascorbate plus 3 m*M* glutathione) showed a significantly ($p < 0.01$) lower TBARS concentration (109 ± 3) than the 3 m*M* ascorbate (without glutathione)-treated group (172 ± 27) (Fig. 4). When pancreatic tissue slices were treated with 3 m*M* dehydroascorbate, TBARS concentration was ele-

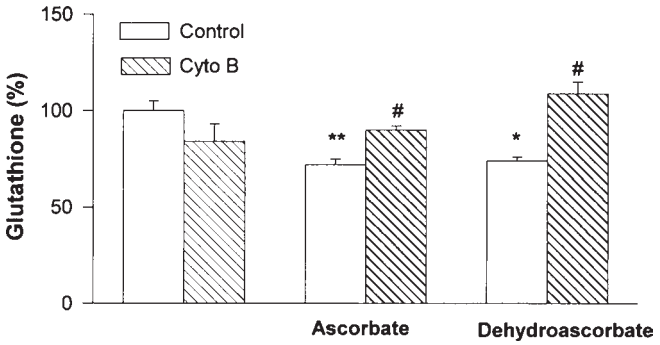


Fig. 3. Effects of ascorbate and dehydroascorbate with (▨) or without (□) cytochalasin B (Cyto B) on glutathione concentrations. Each vertical bar represents the mean ± SEM. Six independent experiments were performed. $*p < 0.05$; $**p < 0.01$; $^{\#}p < 0.05$ between presence and absence of Cyto B.

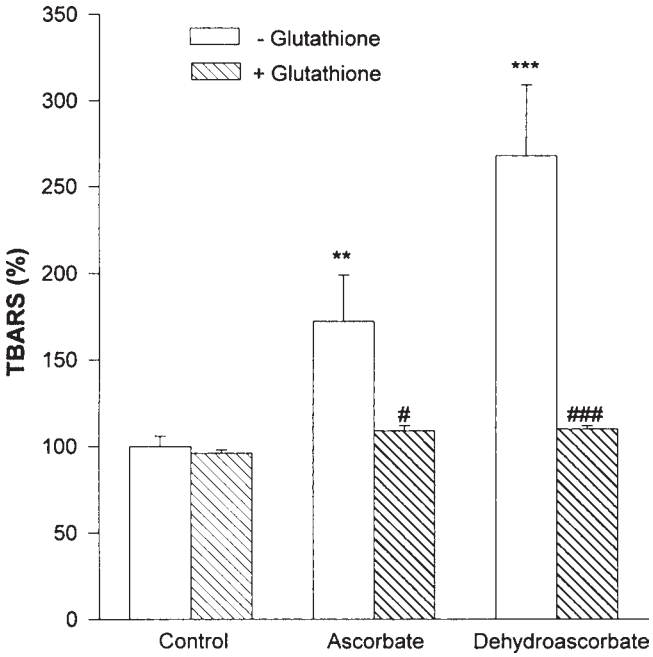


Fig. 4. Effects of ascorbate and dehydroascorbate with (+Glutathione) or without glutathione (–Glutathione) on TBARS production. Quantities are expressed in percentages of control values. Each vertical bar represents the mean ± SEM. Six independent experiments were performed. $**p < 0.01$; $***p < 0.001$; $^{\#}p < 0.05$; $^{\#\#\#}p < 0.001$ between presence and absence of glutathione.

vated to 268 ± 41%. However, the stimulatory effect of dehydroascorbate was ineffective (111 ± 2; $p < 0.001$) when glutathione was added to medium containing dehydroascorbate (3 m*M* dehydroascorbate plus 3 m*M* glutathione) (Fig. 4).

The islet cells from the control group (Fig. 5A) appear as compact groups of pale-staining cells surrounded by acini. Following treatment with 1.0 m*M* ascorbate (Fig. 5B) or 3.0 m*M* ascorbate (Fig. 5C), the islets appeared to be less compact in appearance owing to the presence of more connective-like tissue between the granular cells. Following treatment with 3 m*M* ascorbate plus 0.3 m*M* cytochalasin B (Fig. 5D), the islets appeared like those of the control.

Discussion

Ascorbate cannot be synthesized in human and guinea pig owing to lack of the enzyme L-gulono-γ-lactone oxidase *(13)*. Therefore, ascorbate in humans is solely acquired by absorption from external sources. Ascorbate is easily oxidized to dehydroascorbate in solution, and intestine may absorb substantial quantities of ascorbate as dehydroascorbate via GLUT *(14)*. Ascorbate in plasma (0.1 m*M*) *(15)* is transported into cells so that large amounts of ascorbate can accumulate in particular organs such as the adrenal glands (15–20 m*M*), liver (1 m*M*), brain (2 to 3 m*M*), and pancreas (1 m*M*) *(4,16)*. Three different mechanisms of ascorbate transportation into cells are known: sodium-dependent ascorbate transporter (SVCT) *(17)*, glutamate-ascorbate heteroexchange *(6,18–20)* and GLUT *(7,11,12)*. The first two routes transport ascorbate with no accompanying oxidation or reduction action. However, the third route takes up dehydroascorbate, which is reduced back to ascorbate in intracellular fluid. The reduction processes will generate oxidative stress in the cells. Although ascorbate is a well-known vitamin, only a few physiologic functions have been established.

Ascorbate is known to be a cofactor for a variety of hydrolases that induce hydroxylation of proline residues within procollagen *(21)*. Hydroxylation of the proline is essential for the proper arrangement of the collagen molecule *(22)*. The complete lack of ascorbate in the diet results in poor helical formation and poorly crosslinked strands in collagen and can lead to disease such as scurvy *(23)*. Ascorbate is also an important enzyme cofactor that stimulates transportation, translation, and posttranslation processing of various proteins *(24)*. However, the physiologic roles of ascorbate in those ascorbate-rich organs have not been closely examined, and thus the significance of ascorbate in the pancreas is unknown.

We have been trying to establish the mechanism of ascorbate-induced oxidative stress since we observed that ascorbate killed PC12 cells during our study of ascorbate's potentiation on dopamine action *(1)*. We have shown that ascorbate generates oxidative stress by ascorbate recycling processes in neuronal cells (PC12 and the brain) *(2,3)*. High uptake rates of dehydroascorbate via a carrier mechanism (GLUT) subjugate defensive mechanisms against oxidative compounds such as glutathione and tocopherol and then strip electrons from vital chemicals in the cells causing oxidative stress. In the present study, oxidative stress is represented by elevation of TBARS concentrations and decrease in glutathione concentrations.

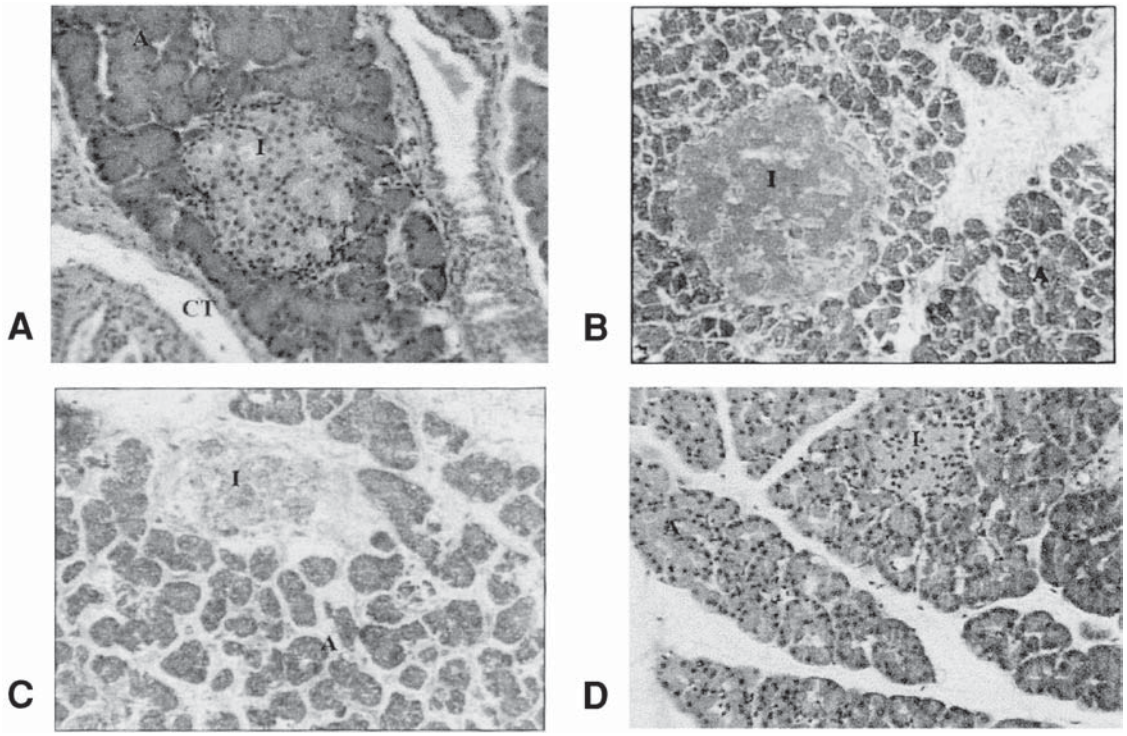


Fig. 5. Light microphotographs of rat pancreas showing Langerhans islets (I) surrounded by acini (A). The pancreatic tissues were treated as follows: (**A**) control (immediately after dissection) without any treatment, (**B**) 1 m*M* ascorbate, (**C**) 3 m*M* ascorbate, and (**D**) 3 m*M* ascorbate and 0.3 m*M* cytochalasin. CT, connective tissue. Magnification ×100.

Ascorbate generates oxidative action in the presence of transition metals such as ferrous ion (Fenton reaction) *(25,26)*. It is possible that the ascorbate-induced oxidative stress in a tissue can be generated by the Fenton reaction since ferrous ion is ubiquitously present in the body. However, the majority of experimental evidence for interactions between ferrous ion and ascorbate is derived from cell homogenates in salt solution such as phosphate-buffered saline (PBS). When we tested the effect of ferrous ion on ascorbate-induced oxidative action, the potentiation action of the ferrous ion was confirmed with cell homogenate in PBS but not in culture medium containing fetal calf serum (FCS), which in the latter is likely owing to inactivation of ferrous ion by interaction with protein in FCS *(27)*. These observations indicated that the ascorbate-induced oxidative stress in the present study is not generated by interactions between ascorbate and ferrous ion.

To support our hypothesis of ascorbate recycling, we have compared the ascorbate-induced oxidative stress between live cells and cell homogenate. Ascorbate consistently oxidized cellular components in the live cells but was ineffective in cell homogenate *(28)*. The differential effects between cells and cell homogenate support our hypothesis that oxidized ascorbate (dehydroascorbate) should be transported into the cells and then reduced back to ascorbate inside the cells that oxidize cellular components.

Glutathione is an endogenous protective agent against oxidative stress. Ascorbate-reduced glutathione concentrations before production rates of lipid peroxide were significantly elevated (1.0 m*M* ascorbate in Figs. 1 and 2), suggesting that ascorbate should consume a substantial amount of glutathione before lipid peroxide concentrations are increased. The glutathione can protect ascorbate against oxidation in medium, and it is known that it cannot penetrate cell membranes. We have confirmed that the glutathione in the medium had no effect on oxidative stress generated inside cells in our experimental conditions *(2)*. When we added glutathione in the culture medium, ascorbate was unable to increase TBARS concentration (Fig. 4). This observation suggests that glutathione prevents oxidation of ascorbate to dehydroascorbate and rapidly reduced dehydroascorbate back to ascorbate. Our observations support the fact that ascorbate cannot generate oxidative stress unless ascorbate is oxidized to dehydroascorbate, which will be transported into cells.

Our former study was limited to neuronal cells. Pancreatic slices were used in the present study to expand our hypothesis that ascorbate recycling generates oxidative stress triggering apoptosis. It is well known that pancreas contains a high concentration of ascorbate that cannot be maintained during adverse conditions such as interruption of blood supply so that the ascorbate will be released into extracellular

fluid. The released ascorbate is likely oxidized to dehydroascorbate since blood carries sufficient amounts of free oxygen (Pa_{O_2} 95 ± 2 torr) *(29)* to oxidize ascorbate. When ascorbate is depleted in the cells, they will try to activate uptake mechanisms in order to restore the normal concentration (homeostasis) of ascorbate in pancreas. There is a good possibility of mobilization of GLUT in storage sites (inactive form) to membrane (active form), which thus increases the transportation capacity of dehydroascorbate, which, in turn, generates oxidative stress thereby triggering apoptosis. Cytochalasin B inhibits transportation of glucose and dehydroascorbate via GLUT *(30,31)*. When we blocked the mobilization of GLUT in storage sites by treating pancreatic tissue with cytochalasin B, ascorbate-induced oxidative stress was blocked, supporting our ascorbate-recycling hypothesis. Oxidative stress in pancreas does not implicate that endocrine cells may be affected since the number of endocrine cells in the pancreas is a small fraction. We therefore examined islets using light microscopy. The islets appeared less compact following ascorbate treatment but following cytochalasin B resembled the islet from untreated control.

These observations may suggest that the recycling of ascorbate may affect the organization of the islets and possibly the individual cells as well. Future studies using immunocytochemistry and electron microscopy will further clarify these observations. We propose a hypothesis that interruption of blood supply might cause damage of endocrine cells via ascorbate-recyling processes.

Materials and Methods

Animals

Male Sprague-Dawley rats (250–300 g) were purchased from Charles River Canada (Montreal, Canada). The rats were allowed to acclimate at 25ºC and a 12-h light-dark cycle (lights on at 8:00 AM) for 1 wk. The rats were given Purina rat chow and water ad libitum. The experimental procedures were approved by Queen's University Animal Care Committee.

Sample Collection

The rats were killed by decapitation under halothane anesthetic and their entire pancreases were removed. The pancreases were sliced (<1 mm) with a razor blade, and 200 mg of tissue/beaker was then transferred to a Teflon beaker containing 2.95 mL of culture medium (RPMI 1640 [Bio-Rad, Hercules, CA] plus 10% horse serum [Gibco]). Final concentrations of 0, 0.3, 1.0, or 3.0 m*M* ascorbate or dehydroascorbate were added to the beaker containing pancreatic tissue. The samples were incubated in a water-jacketed incubator (Forma Scientific) at 37ºC for 3 h under a water-saturated atmosphere of 5% CO_2–95% air. Following the incubation period, the samples were centrifuged for 4 min at 1000*g*. The medium was discarded. Three milliliters of PBS was added to the pellet, which was suspended with a vortex and recentrifuged. The tissue (200 mg) was homogenized in 2 mL of 4.4 m*M* butylated hydroxytoluen (BHT) with a frosted-glass tube-pestle.

Measurement of TBARS Concentration

The quantities of TBARS were analyzed to determine the quantities of lipid peroxides in the pancreatic tissues. To 0.6 mL of homogenate, 0.2 mL of 8.1% sodium dodecyl sulfate (SDS), 50 µL of l 0.8% BHT–acetic acid, 1.5 mL of acetic acid buffer (20 mL of acetic acid + 80 mL of water, pH 3.5), 3.0 mL of 0.8% thiobarbituric acid, and 0.7 mL of distilled water were added. The samples were mixed using a vortex and heated for 1 h in a boiling hot water bath and then cooled in an ice bath. The test tubes were centrifuged at 1000*g* for 2 min. An ultraviolet-visible spectrophotometer (Shimadzu) was used to measure optical density of the samples at 532 nm. The concentrations of TBARS were calculated using tetraethoxy propane (Sigma, St. Louis, MO) as reference ($r = 0.9889$). The quantities were expressed in percentage of control (100%) to compensate for the variation in absolute weights of lipid peroxides among different experiments.

Measurement of Glutathione Concentration

Glutathione concentration was determined in the pancreatic slices. To 0.2 mL of 5% homogenate, 0.8 mL of 25% sodium metaphosphorate (Na_2HPO_3) and 3.0 mL of 0.1 *M* sodium phosphate–0.005 EDTA (pH 8.0) were added and mixed using a vortex. One milliliter of the solution was transferred to a 2-mL Eppendorf tube and centrifuged at 4ºC for 30 min at 14,000*g*. After centrifugation, 0.1 mL of the supernatant was diluted with 1.8 mL of EDTA and 0.1 mL of 0.1% *o*-phthaldialdehyde (4 mg + 4 mL of methanol). Glutathione (Sigma) was used as a reference standard. The reactant was measured at 420 nm (emission, excitation wavelength; 350 nm). Glutathione levels were expressed in percentage of control (100%).

Pancreatic Sample Preparation

Pancreatic tissue from control and treated tissues was fixed by immersion in 2.5% glutaraldehyde in 0.1 *M* sodium cacodylate buffer (pH 7.4) at 4ºC overnight. The samples were then postfixed with 1% osmium tetroxide for 2 h in ice and dehydrated by gradually increasing the concentration of acetone. The samples were then embedded in Epon 812 for 4 h and transferred to capsules and incubated for 48 h at 60ºC. One-micrometer-thick sections were cut with a glass knife on an ultramicrotome and then mounted on glass microscope slides. The samples were stained with 1% toluidine blue or hematoxylin and eosin.

Statistical Analyses

Graph-Pad Prism 2.0 (Graph-Pad, San Diego, CA) was used for statistical analyses. Differences among groups were assessed by one-way analysis of variance with Bonferroni post-tests. Data were presented as the mean ± SEM. A difference of $p < 0.05$ was considered significant.

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