

Free Radicals and the Pathogenesis of Type 1 Diabetes

β -Cell Cytokine-Mediated Free Radical Generation Via Cyclooxygenase-2

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Free radical formation evoked by proinflammatory cytokines has been suggested to be involved in the destruction of β -cells in the course of type 1 diabetes development. However, there is no direct evidence to support this hypothesis. In this study, we used electron paramagnetic resonance spectroscopy in conjunction with spin-trapping methodology to directly determine whether cytokines give rise to free radical formation in the islets. Our results demonstrate that direct, in vivo administration of tumor necrosis factor- α (1,000 units), interleukin-1 β (1,000 units), and interferon- γ (2,000 units) into the rat pancreas through a bile duct cannula leads to the formation of lipid-derived free radicals in this tissue. These free radicals most likely are generated by the β -cells because previous depletion of these cells by streptozotocin abolished the cytokine-induced free radical formation. Furthermore, macrophage depletion was found to decrease the production of free radicals. Inhibition of the enzyme inducible cyclooxygenase (COX-2) and the transcription factor nuclear factor- κ B (NF- κ B) significantly diminished the free radicals' signal intensity, implicating these factors in the formation of free radicals. We have also demonstrated that cytokine treatment leads to the activation of NF- κ B in the pancreatic islets of the rats. *Diabetes* 52: 1994–1999, 2003

There are numerous studies showing that proinflammatory cytokines interleukin (IL)-1 β , interferon (IFN)- γ , and tumor necrosis factor- α (TNF- α) are critically involved in the pathogenesis of type 1 diabetes (1). For instance, it has been shown that IL-1, IFN- γ , and TNF- α synergistically impair β -cell function and cause β -cell death when incubated with isolated rat islets (2). Furthermore, a variety of cytokines have been detected in the insulinitis lesions of NOD mice and BB rats, two animal models of autoimmune diabetes

(1,3). Immunoneutralization of IL-1, IL-6, and IFN- γ with corresponding antibodies or soluble receptors has been effective in reducing the incidence of diabetes in NOD mice (4–7). Transgenic expression of IFN- γ (8) or IFN- α (9) in mouse β -cells led to the induction of insulinitis and diabetes. The molecular mechanisms responsible for the cytotoxic effects of these cytokines, however, remain to be elucidated. Free radicals, particularly reactive oxygen species (ROS), have been implicated in the cytokine-mediated islet cell injury, mostly on the basis of the protective effect of antioxidants in different models of type 1 diabetes. For instance, administration of desferrioxamine, which prevents formation of hydroxyl radicals, protects the mice from streptozotocin (STZ)-induced insulinitis and hyperglycemia (10). Administration of the antioxidant enzymes superoxide dismutase (SOD) and catalase prevented destruction of islet allografts in NOD mice (11). Furthermore, the antioxidant probucol was shown to reduce the diabetes incidence and to delay diabetes onset in the BB rats (12). We have demonstrated that chronic administration of the free radical scavenger phenyl-N-tert-butyl nitron (PBN) inhibits STZ-induced diabetes in mice (13). The low level of antioxidant enzymes such as SOD, catalase, and glutathione peroxidase in the islets is another indication that β -cells are exceptionally vulnerable to oxidative damage (14).

ROS generation, evidenced by the formation of lipid peroxidation products, is believed to be the ultimate cause of cytokine-mediated death of β -cells in isolated islets (15). In addition, IL-1 has been found to cause the expression of heme oxygenase and heat shock protein 70 in isolated islets, an expression pattern that is the characteristic response to oxidative stress (16). No such response was observed in other tissues (16). IL-1 was also found to induce the expression of the antioxidant enzyme manganese SOD in the islets, suggesting IL-1-mediated oxidative stress in the islets (17). However, despite this circumstantial evidence in support of free radical formation in the islets, no direct evidence of the cytokine-mediated generation of these species has been provided. Electron paramagnetic resonance (EPR) spectroscopy is a unique technique by which free radicals can be detected directly, but previous attempts by another group of investigators to detect free radicals in the cytokine-treated isolated islets using this technique did not meet with success (18). Thus, the major objective of this study was to demonstrate the formation of free radicals in the islets as a result of cytokine treatment using EPR spectroscopy.

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COX, cyclooxygenase; EPR, electron paramagnetic resonance; HBSS, Hank's balanced salt solution; IFN, interferon; IL, interleukin; NF- κ B, nuclear factor- κ B; PBN, phenyl-N-tert-butyl nitron; PDTC, Pyrrolidine dithiocarbamate; PGE₂, prostaglandin E₂; ROS, reactive oxygen species; SOD, superoxide dismutase; STZ, streptozotocin; TNF- α , tumor necrosis factor- α .

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It is now well established that activation of the transcription factor nuclear factor- κ B (NF- κ B) is a crucial trigger of inflammatory responses (19–21). The inflammatory enzyme inducible cyclooxygenase (COX-2) is among the proteins that are expressed as a result of NF- κ B activation (22). Free radicals have been found to be generated as a result of the activity of this enzyme (23,24). In this study, we investigated the possible involvement of NF- κ B and COX-2 in the formation of the cytokine-induced free radicals using inhibitors of these factors and immunohistochemical analysis.

RESEARCH DESIGN AND METHODS

Animals. Wistar rats (male, 8–10 weeks old) were fasted overnight. The next morning, the rats were anesthetized using an isoflurane-oxygen mixture and the bile duct was cannulated at the duct of pancreas entering the duodenum, via an incision made through the skin in the upper left quadrant, posterior to the xyphoid process into the peritoneal cavity. The cannula entered the pancreas through the pancreatic duodenal duct. A ligature was placed just below the biliary tree, preventing bile flow into the pancreas, thus isolating the pancreas from biliary interferences. Once the cannula was in place, 0.5 ml of Hank's balanced salt solution (HBSS) was injected into the pancreas, followed by 1 ml of HBSS containing the cytokines (rat recombinant IL-1, 1,000 units; IFN- γ , 2,000 units; TNF- α , 1,000 units; R&D Systems, Minneapolis, MN). After the injection, the incision was closed, leaving the cannula projecting out of the abdominal cavity. Rats were then treated with buprenorphine hydrochloride (3 mg/kg, intraperitoneal injection) to relieve the postoperative pain. Five hours after treatment, the rats were once again anesthetized, and the spin-trapping agent, PBN (150 mmol/l in 25 mmol/l Tris-HCl buffer [pH 7.4], 1 ml), was injected into the pancreas through the bile-duct cannula and allowed to trap the formed radicals for 1 h. In experiments designed to test the effect of β -cell depletion on free radical formation, rats were fasted overnight and injected with STZ (80 mg/kg; Sigma Chemical Co., St. Louis, MO) via intravenous injection into the tail vein under anesthesia the next morning. β -Cell depletion was confirmed by the measurement of blood glucose level (tail vein) using the Accu-Check Advantage glucometer 48 h after STZ injection. Cytokine treatment in these rats was performed 4–6 days after the STZ administration. To deplete macrophages, rats were treated with gadolinium (III) chloride (10 mg/kg) through intravenous injection 20 h before cytokine administration (25). In experiments in which different inhibitors were used, they were administered via intraperitoneal injections 20 min before cytokines. The COX-2 inhibitor NS-398 (Cayman Chemical, Ann Arbor, MI) was dissolved in 100 μ l of 50% DMSO (Sigma Chemical Co.) and administered via intraperitoneal injection (2 mg/rat). Pyrrolidine dithiocarbamate (PDTC) was dissolved in 100 μ l of saline and injected directly into the pancreas (4 mg/rat) via the bile duct cannula.

EPR spectroscopy. For performing EPR spectroscopy, the pancreas was removed, washed with normal saline, cut into small pieces, and placed in 5 vol of 25 mmol/l Tris buffer (pH 7.4). Pancreatic tissue was homogenized using a Potter-Elvehjem tissue grinder with a motor-driven pestle. The homogenate was extracted with 10 vol of high-performance liquid chromatography grade chloroform and was subsequently evaporated to dryness using a rotary evaporator. The dried extract was resuspended in 650 μ l of toluene, placed in a quartz round EPR cell, and purged with nitrogen for 15 min. For ensuring that no artifact free radicals were introduced into the sample as a result of the extraction process, the used PBN was put through the same extraction procedure (in the absence of the sample) and its EPR spectrum was recorded before each experiment. EPR analysis was performed on a Bruker 300E spectrometer (Reinstetten, Germany) using the following conditions: modulation amplitude, 1.0 G; modulation frequency, 100 KHz; microwave power, 20 mW; conversion time, 92 ms; sweep time, 84 s. Control rats underwent the complete sham procedure but received only the vehicle (HBSS) in place of the cytokine solution. All of these procedures were performed in accordance with the guidelines for the use and care of laboratory animals of Oklahoma Medical Research Foundation.

Islet immunohistochemistry. For immunohistochemical analysis of NF- κ B activation, rats were treated with cytokines or vehicle (control), as described above. Thirty or 60 min after the treatment, pancreas was perfused with paraformaldehyde (4% in PBS) and removed. Immunohistochemistry was performed on cryosectioned tissue (8 μ). Briefly, frozen tissue slices were thawed and incubated in cold acetone for 5 min. The tissue slides were then rinsed with PBS (2 \times 5 min) and were subsequently incubated with hydrogen peroxide (3% in PBS) for 5 min. After two rinses with PBS (2 \times 5 min), the

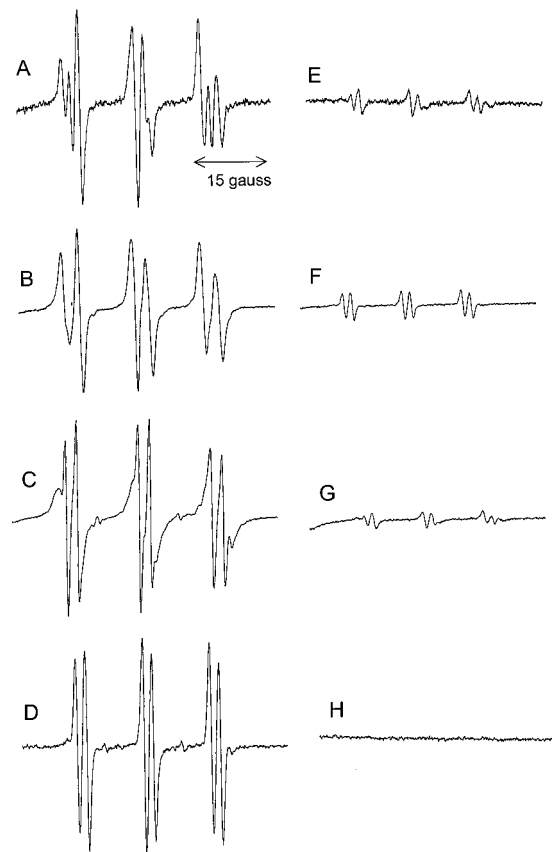


FIG. 1. EPR spectra of spin-trapped free radicals recorded from rat pancreatic extracts after *in vivo* intrapancreatic treatment with cytokines (A–D) or vehicle (E–H). Each spectrum represents an individual animal.

nonspecific binding in the tissue was blocked using BSA (4%) containing normal rabbit serum (0.4%) for 15 min, followed by the avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA). Next, the tissue slices were incubated with goat anti-NF- κ B p-65 antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) for 1 h in a humidifying chamber. After two 5-min rinses with PBS, the slides were treated with the secondary antibody, biotinylated rabbit anti-goat IgG (Vector Laboratories) for 1 h (in the humidifying chamber). After two rinses with PBS (2 \times 5 min), the slides were treated with the Vectastain ABC kit (Vector Laboratories) for amplification of the colorimetric reaction. 3-Amino-9-ethylcarbazole (Vector Laboratories) was used as the substrate for the peroxidase-catalyzed colorimetric reaction. Microscopic images were recorded using a Zeiss Axioplan 2 microscope (Thornwood, NY). Insulin immunohistochemistry was performed using a mouse monoclonal insulin antibody from NeoMarkers (Fremont, CA) following the same procedure.

Statistical analyses. One-way ANOVA followed by Student's *t* test was used to determine the statistical significance of the differences among the obtained values.

RESULTS

Free radical formation as a result of *in vivo* cytokine administration to the pancreas. EPR spectroscopy in conjunction with PBN spin trapping demonstrated that direct administration of proinflammatory cytokines IL-1, IFN- γ , and TNF- α to the pancreas under *in vivo* conditions leads to the formation of free radicals in this organ, as demonstrated in Fig. 1 ($n = 6$). In EPR spin-trapping method, free radicals produced in the tissue are trapped and stabilized by a spin-trapping agent to allow one to record its EPR spectrum *ex vivo* (26). When more hydrophilic spin trap 5,5'-dimethylpyrroline-N-oxide (27) was used as the spin trap, no free radicals were detected.

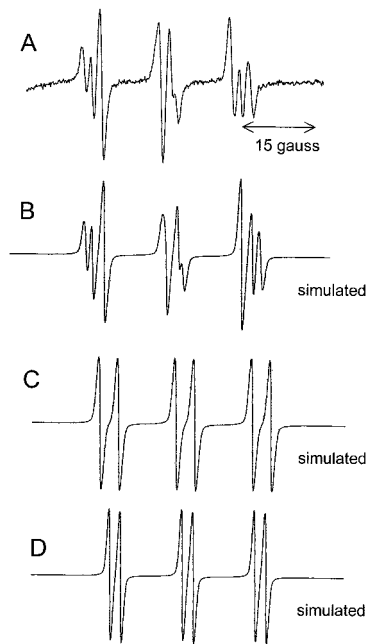


FIG. 2. Computer spectral simulation of the EPR spectrum of the spin-trapped free radicals in pancreatic tissue. *A*: Actual EPR spectrum for spin-trapped free radicals (same as Fig. 1*A*). *B*: A computer-simulated EPR spectrum reproducing the EPR spectrum *A*. EPR parameters used for the spectral simulation were as follows: free radical 1 (spectrum *C* assigned to *L*): $A(N) = 15.3$ gauss, $A(H) = 3.8$ gauss, line width = 1.0 gauss, and free radical 2 (spectrum *D* assigned to *LO*): $A(N) = 14.6$ gauss, $A(H) = 2.2$ gauss, line width = 0.8 gauss. For producing the composite spectrum *B*, the individual spectra calculated for free radicals 1 (*C*) and 2 (*D*) were superimposed with a ratio of 52:48 and the center of the spectrum *C* was shifted 0.1 gauss to the lower field (*left side*). The highest field lines (*right side lines*) in the actual spectrum (*A*) are broadened (shortened) by motional effect characteristic of solution EPR spectra. Other spectra shown in Figs. 1*B–D* can be readily reproduced by changing the ratio of superposition of *C* and *D*.

However, when PBN was used as the spin-trapping agent, we were able to detect cytokine-induced free radical formation, suggesting that radicals within the lipid-rich membrane or possibly lipoproteins in the pancreatic tissue were trapped by the more lipophilic, cell membrane-penetrable PBN. A negligible amount of free radical formation was observed in the pancreatic tissue of the vehicle-treated animals ($n = 6$; Fig. 1). The complexity of the splitting patterns in the obtained spectra, however, indicated the existence of more than one radical species trapped by PBN. EPR spectra were reproduced with computer spectral simulation software (O 61 zone Software; Guelph, Ontario, Canada). Using computer EPR spectral simulation in addition to the observed hyperfine splitting constants (spectroscopic constants obtained from the spectrum) the PBN-trapped radicals were assigned to carbon-centered radicals of lipid [$\cdot\text{CH}_2(\text{CH}_2)_n\text{CH}_2\text{COOR}$] and oxygen-centered radicals of lipoxyl origin [$\cdot\text{OCH}_2(\text{CH}_2)_n\text{CH}_2\text{COOR}$] (28). The hyperfine splitting constants of the simulated spectra, shown in the legend of Fig. 2, further confirm the assignment of the radicals as lipid and lipoxyl radicals. EPR spectra obtained from the tissue shown in Fig. 1 can be reproduced by superimposing the two simulated free radical species with various concentration ratios.

Effect of β -cell depletion on cytokine-induced free radical formation. For determining whether β -cells con-

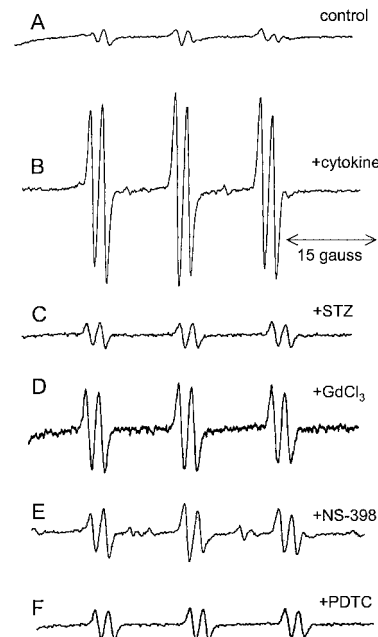


FIG. 3. Effect of various treatments on EPR spectral intensity (free radical level). EPR spectra of spin-trapped free radicals were recorded in the pancreatic extract obtained from rats after in vivo intrapancreatic cytokine administration with pre- or posttreatment with various agents and inhibitors. *A*: Cytokines alone, no inhibitor. *B*: Pretreatment with STZ to deplete β -cell. *C*: Pretreatment with gadolinium chloride to deplete macrophages. *D*: Posttreatment with NS-398 to inhibit COX-2 activity. *E*: Pretreatment with PDTC to inhibit NF- κ B activation.

tributed to the cytokine-induced formation of free radicals, in a separate experiment rats ($n = 5$) were treated with the selective β -cell toxin STZ (29) before cytokine administration. STZ-induced β -cell destruction was confirmed by measurement of the blood glucose level of the treated rats. The rats treated as described had blood glucose levels >350 mg/dl. Cytokine administration was performed 4–6 days after STZ administration. EPR spectra obtained from the pancreatic tissue of these animals depicted no or very weak signals (Fig. 3).

Effect of macrophage depletion on the generation of free radicals. For determining the contribution of macrophages, which may infiltrate the pancreas as a result of cytokine administration (i.e., inflammatory reaction), to the formation of free radicals, the effect of macrophage depletion on this process was studied. When macrophages were depleted by previous treatment of the rats with gadolinium chloride ($n = 4$), free radical generation was still observed after the administration of cytokines, but the treatment diminished the degree of free radical formation by $\sim 50\%$ (Figs. 3 and 4).

Effect of the inhibition of COX-2 on cytokine-induced free radical generation. Cytokines have been shown to cause COX-2 expression in isolated islets. COX has been shown to cause free radical generation in the process of arachidonic acid metabolism to prostaglandins and thromboxane (23,30). To determine the possible involvement of COX-2 in the cytokine-mediated formation of free radicals, we investigated the effect of the selective COX-2 inhibitor NS-398 (31) on radical formation. As shown in Fig. 3, pretreatment of the rats with this inhibitor followed by cytokine treatment prevented free radical formation in the

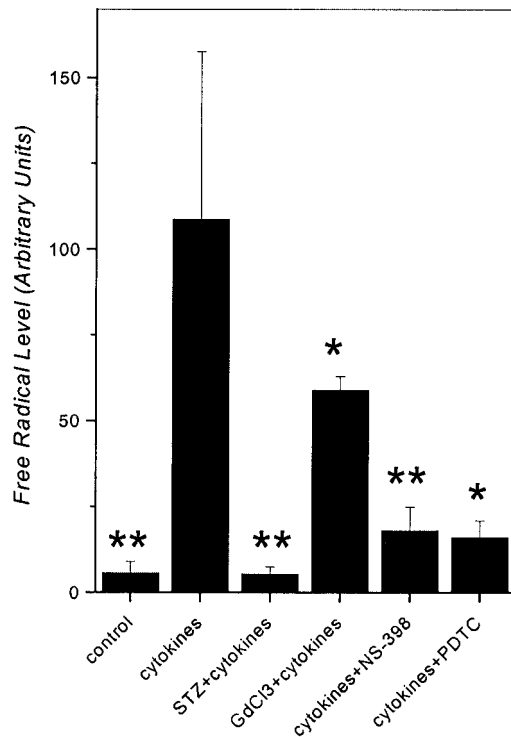


FIG. 4. Free radical levels obtained in the pancreatic tissue after various *in vivo* treatments. Error bars show SD in each treatment ($n = 4-6$). * $P < 0.05$, ** $P < 0.01$ vs. cytokines.

pancreas of the treated animals ($n = 4$), at least partially. Cytokine-induced free radical generation was not suppressed by pretreatment of the rats with NS-398 vehicle, 50% DMSO ($n = 4$).

Islet-specific NF- κ B activation after cytokine administration to the pancreas. Immunohistochemical analyses performed on the pancreatic tissue obtained from control and cytokine-treated rats demonstrated that cytokine administration to the pancreas resulted in the islet-specific activation and translocation of the transcription factor NF- κ B into the nucleus. NF- κ B activation was observed at 30 and 60 min after cytokine treatment (Fig. 5). No NF- κ B activation was observed in the acinar tissue ($n = 4$). NF- κ B activation was no longer present in the rats tested 5 h after the treatment (data not shown). When rats were pretreated with the established NF- κ B inhibitor PDTC (32), cytokines did not evoke islet NF- κ B translocation (data not shown). Administration of the COX-2 inhibitor NS-398, however, did not prevent cytokine-induced NF- κ B activation in the islets (data not shown).

Effect of NF- κ B inhibition on the cytokine-induced free radical generation in the pancreas. Inhibition of

the activation of NF- κ B by means of PDTC pretreatment almost completely abolished free radical generation in the pancreas of the cytokine-treated animals ($n = 4$), as shown in Fig. 3. This finding demonstrates that NF- κ B activation was involved in the generation of the observed free radicals.

DISCUSSION

In this study, we demonstrated for the first time that pancreatic β -cells produce free radicals in response to proinflammatory cytokines. Summarizing the results, we have shown that 1) cytokines evoke a significant level of free radical formation in the pancreas; 2) β -cell depletion before the administration of cytokines annihilates the free radical formation, whereas depletion of macrophages has a partial inhibitory effect; 3) selective inhibition of COX-2 significantly decreases the free radical level; and 4) cytokines induce islet-specific activation of NF- κ B, and inhibition of this transcription factor suppresses free radical formation. Detection of the formed radicals was made possible by the use of EPR spectroscopy in conjunction with spin-trapping technique. EPR spectroscopy is the only available method for the direct detection of free radicals. However, most free radicals are extremely reactive and short-lived, qualities that render their direct detection tremendously difficult. The spin-trapping method, in which the unstable free radical reacts with a trap molecule (spin trap) to form a more stable, long-lived radical species, allows the detection of many otherwise undetectable radicals (28,33). In this study, we took advantage of the ability of the spin-trapping agent PBN to detect cytokine-induced free radical formation in the pancreas of the rats.

EPR spectra obtained from the animals that received cytokines intrapancreatically exhibited the presence of a high level of at least one free radical species in this organ. In most cases, however, the presence of more than one radical was apparent from the recorded spectra. The radicals detected in this study have hyperfine splitting constants characteristic of lipid ($L\cdot$) or lipoxyl radicals ($LO\cdot$) (28). Both of these species may arise from peroxidation of the membrane lipids, a process most likely initiated by ROS. The initially produced ROS, formed through the action of COX-2 (*vide infra*), can abstract a hydrogen atom from a lipid molecule and thus produce the lipid radical ($L\cdot$). Lipoxyl radicals form as a result of metal-ion decomposition of lipid hydroperoxides generated through the reaction of $L\cdot$ with molecular oxygen.

For determining whether pancreatic β -cells play a role in the formation of the observed radicals, they were depleted in a group of rats before their treatment with the

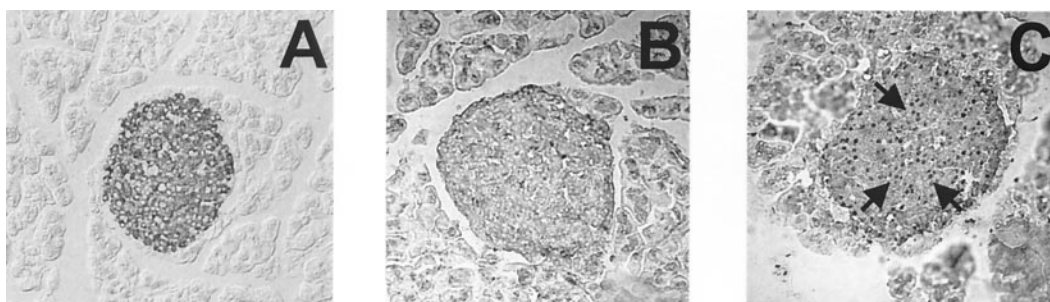


FIG. 5. Immunostaining of insulin in a pancreatic islet (control; A), NF- κ B p65 in a pancreatic islet of a control (vehicle-treated) rat (B), and NF- κ B p65 in a pancreatic islet of a rat (C) treated with cytokines for 60 min. Magnification 100 \times . Arrows point to the nuclear localization of NF- κ B p65 in the islet.

cytokines. Cytokine administration to these rats generated little or no signal, indicating that β -cells serve as the origin of the detected free radicals. This finding is of extreme importance to gaining a better understanding of the mechanisms involved in the pathogenesis of type 1 diabetes, because it demonstrates for the first time that cytokines elicit free radical formation by the β -cells. However, depletion of macrophages by previous gadolinium chloride treatment only partially inhibited the generation of free radicals. The observations that β -cell depletion leads to near total suppression of free radicals, whereas macrophage eradication only partially inhibits this process, indicate that although macrophages contribute to the formation of the detected radicals, their role is indirect. We believe that macrophages most likely enhance the generation of free radicals by the β -cells through the release of additional cytokines.

Our previous studies in the same model demonstrated that in vivo cytokine administration to the rat pancreas leads to the expression of the inflammatory enzyme COX-2 in the islets isolated from the pancreata treated in vivo with cytokines (34). Also, in isolated rat islets, IL-1 has been shown to promote COX-2 expression and prostaglandin E₂ (PGE₂) formation (35,36). Consequently, COX-2 has been proposed to play a role in the pathogenesis of type 1 diabetes (37). Furthermore, we have previously reported that selective inhibition of COX-2 protects mice from low-dose STZ-induced diabetes, pointing to a role for this enzyme in diabetes pathogenesis in this model (38). It is interesting that it has recently been reported that there is an increased expression of COX-2 in patients with established type 1 diabetic and in individuals at higher genetic, immunologic, and familial risk for this disease (39). In addition, inflamed islets of NOD mice have been shown to produce increased levels of COX-2 catalyzed PGE₂ formation (40). Robertson and colleagues (41,42) have reported that unlike most other tissues, in pancreatic islets, COX-2 and not COX-1 (constitutive isoform of COX) is dominantly expressed at the basal level. All of these findings point to a possible link between COX-2 activity and the destruction of the β -cells. Among COX-2 products, PGE₂ is known to inhibit glucose-induced insulin release by β -cells (41,42). IL-1-induced inhibition of insulin secretion by islet 36 cells is mediated by PGE₂ (43). However, like other prostanoids, PGE₂ has both pro- and anti-inflammatory actions (44), and it is not yet clear whether PGE₂ formation by COX-2 in the islets has a protective or detrimental effect.

A more intriguing aspect of COX activity might be that the catalytic activity of this enzyme is accompanied by the formation of ROS (23,24). COX-2 is a complex enzyme with both cyclooxygenase and peroxidase activities. Both of these activities have been shown to give rise to ROS formation (23,24). Our observation that selective inhibition of COX-2 by NS-398 abolishes the cytokine-induced free radical generation suggests that COX-2 activity is indeed the major source of ROS generation in our model. Thus, whereas COX-2-mediated PGE₂ formation may have detrimental consequences for the β -cells, the ROS formation that results from overexpression of COX-2 would undoubtedly damage and destroy the β -cells, particularly

under the chronic inflammatory conditions preceding the death of β -cells.

Proinflammatory cytokines such as TNF- α and IL-1 have been reported to cause the activation of the transcription factor NF- κ B (45,46). The involvement of NF- κ B activation in IL-1-induced PGE₂ formation by isolated rat islets has been demonstrated (35). We have shown that under the experimental conditions used, cytokines evoke the activation and nuclear localization of NF- κ B in the islets and not in the surrounding acinar tissue. We have also demonstrated that inhibition of NF- κ B activation prevents the generation of cytokine-induced free radicals. These findings indicate that cytokine-mediated activation of NF- κ B in the islets leads to generation of free radicals by the β -cells. NF- κ B activation leads to the expression of COX-2, further supporting the notion that COX-2 is likely the major source of free radicals. Considering that the existence of a chronic inflammatory state in and around the islets during the insulinitis phase most likely translates into chronic NF- κ B activation in the islets, these findings demonstrate at least one of the detrimental outcomes of such an event.

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