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Indomethacin Inhibits Thymic Involution in Mice with Streptozotocin-induced Diabetes

Ani A Mic,[1] Felix A Mic,[2,4,*] Calin A Tatu,[1] Mihai Ionac,[3] Valentin L Ordodi,[1] and Virgil Paunescu[1]

Diabetes is chronic disease that is accompanied by a rapid thymus involution. To investigate the factors responsible for thymic involution in a model of STZ-induced diabetes, mice were injected with STZ alone or in combination with the cyclooxygenase 2 inhibitor indomethacin (INDO). Thymus weight, glycemia and serum corticosterone were measured, and apoptosis in thymus and thymocyte cultures was analyzed by flow cytometry. Although earlier studies report that streptozotocin (STZ) is toxic to lymphoid tissues, in our experiments even massive doses of STZ did not negatively affect thymocyte cultures. Cultured thymocytes also seemed unaffected by high glucose concentrations, even after 24 h of exposure. Administration of INDO concomitantly with STZ reduced thymic involution but did not prevent the onset of hyperglycemia or reduce established hyperglycemia. When INDO was given before STZ, the same degree of thymic involution occurred; however, hyperglycemia was reduced, although normoglycemia was not restored. INDO also reduced serum corticosterone. Because thymocytes are known to be sensitive to glucocorticoids, this finding suggests that cyclooxygenase 2 inhibition may retard thymic involution by reducing serum glucocorticoids. In conclusion, our results show that STZ and hyperglycemia are not toxic to thymocytes and that cyclooxygenase 2-mediated mechanisms are involved in thymic involution during diabetes.

Abbreviations: STZ, streptozotocin; INDO, indomethacin; PBS, phosphate-buffered saline

Streptozotocin (STZ)-induced hyperglycemia in mice is an experimental model of diabetes that is useful for gathering information about this disease and its consequences.[13] The hyperglycemia in this model is persistent and leads to complications that mimic some of the manifestations of diabetes in humans.[18,19] Upon induction of diabetes by STZ, the thymus involutes quickly, although the exact mechanism of this involution is unclear. STZ is cleared rapidly from blood, with a half-life of only 15 min,[22] but necrosis of β cells in experimental animals could be detected hours after its administration, the thymus began to involute within 1 d, and hyperglycemia could be demonstrated in 1 to 2 d.[10] Several studies have shown that STZ is toxic to primary lymphoid tissues,[26] impairs the maturation of thymus lymphocytes, and leads to progressive thymic involution by apoptosis of thymocytes.[3,24]

Hyperglycemia in concentrations of 20 to 45 mM induces oxidative stress and apoptosis in cultured dorsal root ganglions.[20] Chronic hyperglycemia activates the stress response that is associated with elevated glucocorticoids,[2,15,23] which are known inducers of apoptosis in thymocytes[5,16] and cause rapid thymic involution in mice. Recent results show that a mild inflammation accompanies type 2 diabetes, and several proinflammatory cytokines (interleukin 1β, tumor necrosis factor α, interferon γ) are involved in the pathogenesis of insulin-dependent diabetes by impairing β-cell function or direct cytotoxicity of these cells.[1,21,] Cyclooxygenase 2 is an essential player in the inflammatory pathway and uses arachidonic acid to synthesize prostaglandin E2, a key inflammatory mediator.[9] Expression of prostaglandin E2 is elevated in patients with insulin-dependent diabetes and persons with familial, genetic, or immunologic risk for disease.[14] Inhibition of cyclooxygenase 2 prevents hyperglycemia in a diabetes model generated through administration of 40 mg/kg STZ daily for 5 consecutive days.[25] Because cyclooxygenase 2 and arachidonic acid metabolites are involved in glucocorticoid-induced thymic involution,[16] the current study aimed to investigate the role of the cyclooxygenase 2 signaling pathway in diabetes-induced thymic involution.

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[1]Department of Physiology, Victor Babes University of Medicine and Pharmacy, Timisoara, Romania; [2]Performa Diagnostics SRL, Timisoara, Romania; [3]Pius Branzeu Center for Laparoscopic Surgery and Microsurgery, Timisoara, Romania; [4]Department of Immunology, Timisoara County Hospital, Timisoara, Romania.
[*]Corresponding author. Email: mic.felix@yahoo.com

Materials and Methods

Animals and in vivo treatments. All animal experiments described herein comply with European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, France, 1986), and the experimental protocol was reviewed and approved by the University of Medicine and Pharmacy Timisoara Board for Animal Experimentation. Throughout the study NMRI mice (age, 4 to 5 wk; weight, 16 to 21 g) were used. The mice were obtained from the Pius Branzeu Center for Laparoscopic Medicine (Timisoara, Romania), which certified them to be free of mycoplasma, adventitious viruses, respiratory and enteric bacteria, and ecto- and endoparasites. The animals were housed under conditions of controlled temperature (21 °C) with a 12:12-h light:dark cycle and had ad libitum access to standard mouse chow and water. STZ was dissolved in citrate buffer (pH 4.5) and injected intraperitoneally within 10 min after preparation. Animals whose glycemia exceeded 11.1 mmol/l (200 mg/dl) at 24 h after treatment were considered diabetic. Indomethacin (INDO) was dissolved in absolute

ethanol and the concentration adjusted so the amount of ethanol injected in animals did not exceed 10 µl/animal and the INDO dose did not exceed 2 mg/kg. All chemicals were purchased from Sigma-Aldrich (St Louis, MO), unless otherwise stated.

Cell culture. The mice were euthanized by ether anesthesia followed by cervical dislocation and their thymuses removed. Thymus tissue was washed with cold phosphate-buffered saline (PBS) to remove blood, minced by passage through sterile wire mesh by using a syringe piston, and resuspended in cold PBS. The cell suspension then was washed twice with PBS and seeded at 10^7 cells/ml in RPMI medium (Sigma) supplemented with 10% fetal calf serum (Gibco, Grand Island, NY), 2 mM glutamine, and 100 IU penicillin; streptozotocin in concentrations of 100 µm and 1 mM, and dexamethasone (1 µM) were added as appropriate. Cells were incubated at 37 °C in an atmosphere of 5% CO_2. STZ was dissolved in PBS and added to cultures, whereas dexamethasone was dissolved in absolute ethanol and added to cultures in concentrations not exceeding 0.1%; control cultures received the corresponding amount of vehicle.

Measurement of thymocytic apoptosis. The cell suspension from thymus was obtained as described in the preceding paragraph and then centrifuged at 200 × *g*; the pellet resuspended in cold 70% ethanol and kept at –20 °C until further processed. Cultured cells were harvested, centrifuged at 200 × *g*, resuspended in cold 70% ethanol, and kept also at –20 °C until further use. For flow cytometry, ethanol-stored cells were centrifuged at 200 × *g* for 5 min, the supernatant was discarded, the cell pellet was washed twice with PBS, and the pellet was resuspended in PBS containing 50 µg/ml propidium iodide and 100 U/ml DNase-free RNase and incubated for 30 min at 4 °C in the dark. The cells then were washed twice with PBS, resuspended in PBS, and analyzed by flow cytometry. Apoptotic thymocytes were visualized as a subdiploid peak, due to DNA fragmentation, on a FACScan flow cytometer (Becton Dickinson Biosciences, San Jose, CA), and the cell populations were analyzed with CellQuest software (Becton Dickinson Biosciences, San Jose, CA).

Corticosterone assay. One set of 20 mice were injected intraperitoneally with 100 mg/kg STZ alone (10 animals) or with 2 mg/kg INDO (10 animals) and euthanized 24 h later. Another group of 20 mice was injected with 100 mg/kg STZ alone (10 animals) or with 2 mg/kg INDO (10 animals, these mice received an additional injection of INDO at 24 h) and euthanized 48 h later. Control animals received the corresponding amount of vehicle. At indicated time points the mice were anesthetized, and blood was drawn by cardiac puncture. The serum was assayed for its corticosterone content by using an enzyme-linked immunosorbent assay kit (DRG Diagnostics, Marburg, Germany) according to the manufacturer's instructions. The results are expressed as ng/ml.

Measurement of glycemia. Glycemia was measured with test strips and a commercial glucose test meter (Achtung glucometer, Taidoc, Taipei, Taiwan) by using whole venous blood obtained by removing a small tail fragment. The animals were fasted for 3 h before glucose measurement, and prior to sampling, tails were anesthetized by dipping in ice-cold ethanol for 10 s.

Statistical analysis. Data are displayed as mean ± standard error from at least 3 independent experiments. Statistical calculations were performed with Gnumeric Spreadsheet (Gnome Office Suite, Gnome Foundation, Cambridge, MA), and groups were compared by using the Student *t* test, and *P* values less than 0.05 were considered significant.

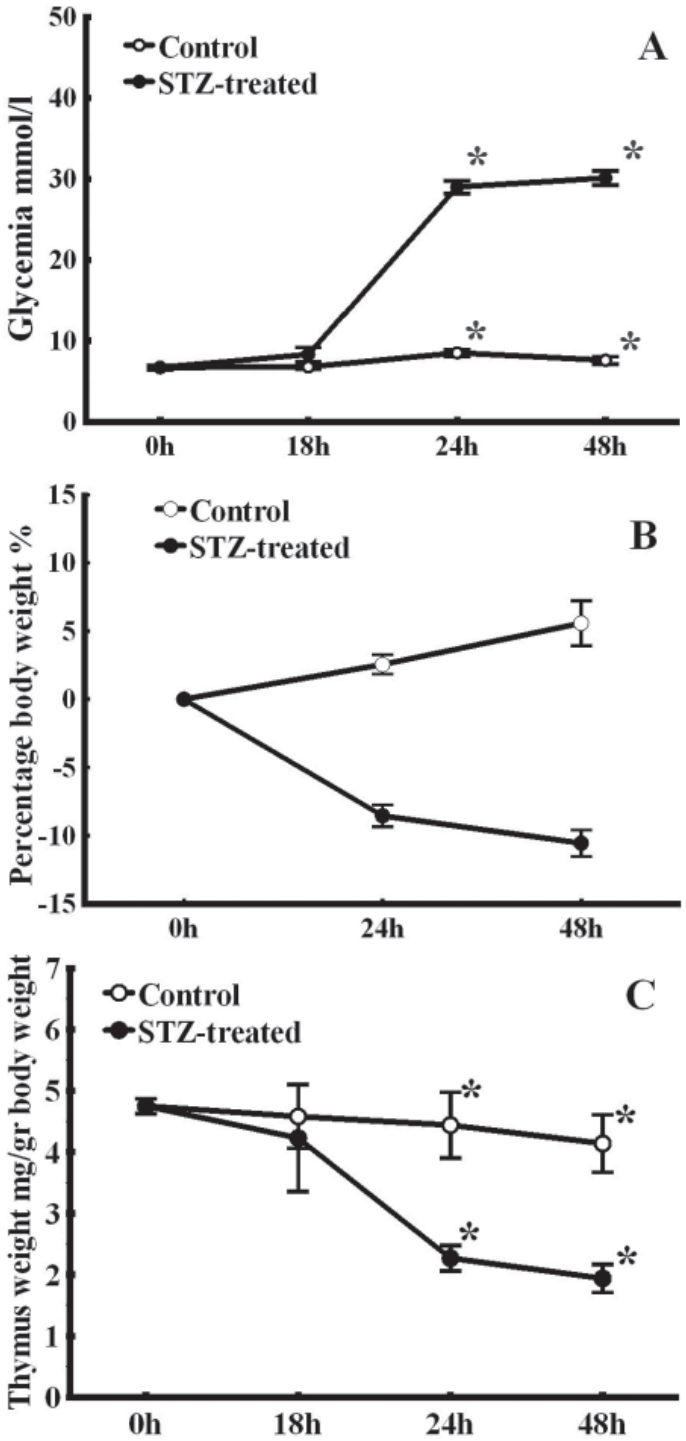

Figure 1. (A) Blood glucose levels in control animals and those treated with a single dose of 100 mg/kg streptozotocin. (B) Body weight evolution in control- and streptozotocin-treated animals. Data is presented as percentage of body weight change per 24 h. (C) Weight of thymus in control- and streptozotocin-treated mice at indicated time points. *, $P < 0.05$.

Results

A single, massive dose of STZ leads to rapid hyperglycemia, thymic involution, and body weight loss. Injection of mice with a single dose of 100 mg/kg STZ led to profound and persistent hyperglyce-

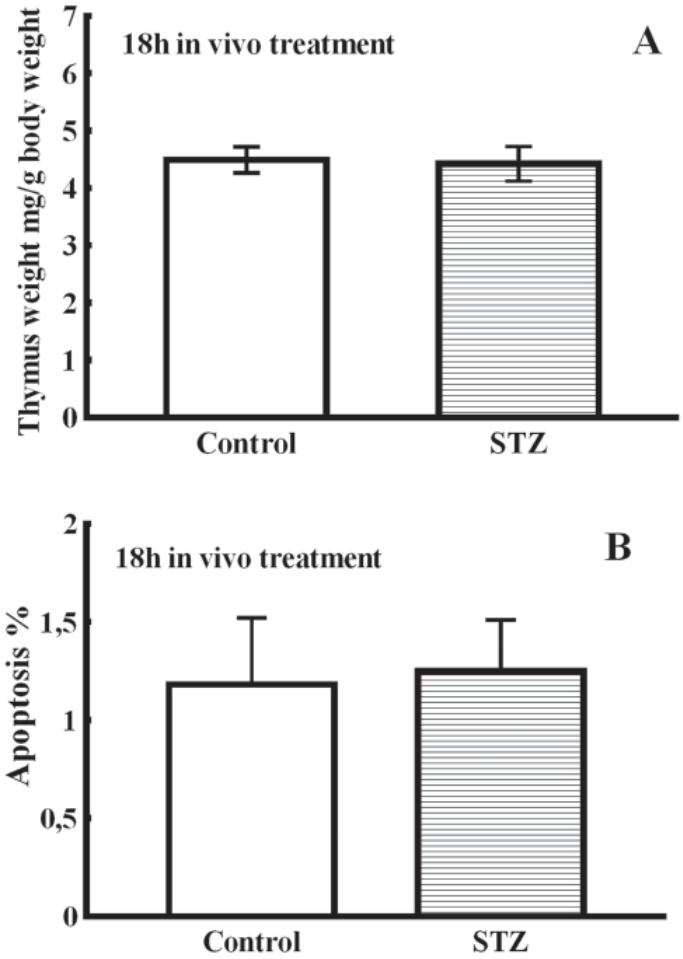

Figure 2. (A) Weight of thymus in control and streptozotocin-treated mice at 18 h after treatment. (B) Apoptosis levels in thymuses 18 h after treatment of control- and streptozotocin-treated mice.

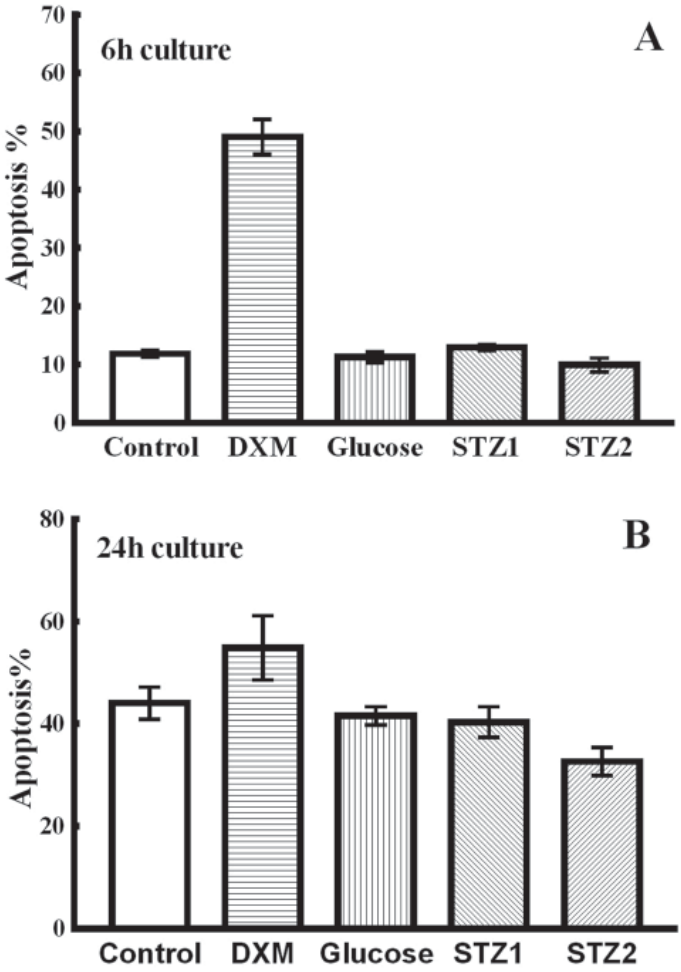

Figure 3. (A) Effect of various concentrations of streptozotocin (STZ1, 100 µM; STZ2, 1 mM), glucose (45 mM), and dexamethasone (1 µM; used as a positive control for thymocytic apoptosis) on thymocytic apoptosis at 6 h after addition of drug to cell cultures. (B) Effect of various concentrations of streptozotocin (STZ1, 100 µM; STZ2, 1 mM), glucose (45 mM), and dexamethasone (1 µM, positive control) on thymocytes apoptosis at 24 h after addition of drug to culture.

mia as early as 24 h after injection (Figure 1 A). The hyperglycemia was long-lasting and associated with rapid body weight loss (Figure 1 B) and dramatic thymic involution (Figure 1 C), consistent with data presented elsewhere in the literature.[3,10]

STZ does not induce directly thymic involution. To assess whether STZ was directly cytotoxic to thymus, a dose of 100 mg/kg was administered and the thymus was weighed 18 h later, before the onset of hyperglycemia. Thymus weight did not differ between vehicle- and STZ-treated mice (Figure 2 A). The thymus weight was expressed as milligram thymus per gram of body weight to compensate for differences in body weight of the animals. Thymic apoptosis, measured by flow cytometry, was comparable in control and STZ-treated thymuses (Figure 2 B). In addition, thymocytes cultured for 6 and 24 h in the presence of 100 µM and 1 mM of STZ revealed no apoptosis, even at the massive dose (Figure 3 A, B).

High glucose is not toxic to thymocytes. Thymocyte cultures were exposed to a high glucose concentration—818 mg/l (45 mmol/l)—for 6 and 24 h. Control thymocytes were cultured in standard RPMI medium (containing 2000 mg/l [11 mmol/l] glucose) for the same time period. The results show that high concentrations of glucose were not detrimental to thymocytes even after 24 h of exposure (Figure 3 A, B). The levels of apoptosis in the thymocytes cultured for 6 and 24 h in the presence of high glucose were comparable to that in control thymocytes cultured with normal levels of glucose. As a positive control, thymocytes were cultured for the same duration with 1 µM dexamethasone, a known inducer of apoptosis in these cells.[16]

INDO inhibits thymic involution and can influence glycemia. Animals were injected concomitantly with 100 mg/kg STZ intraperitoneally and 2 mg/kg INDO (a nonselective cyclooxygenase 2 inhibitor) subcutaneously and euthanized 24 h later. Control animals received the corresponding amount of vehicle. Thymus weight and glycemia were measured in control mice and in the treated ones at the indicated time points. Thymic involution was inhibited significantly ($P < 0.05$) by simultaneous administration of INDO with STZ (Figure 4 A), even when hyperglycemia persisted (Figure 4 B). The rate of apoptosis was lower in thymuses treated with INDO (Figure 4 C) than in thymuses exposed to STZ only, suggesting that inhibition of thymocytic apoptosis retards thymic involution. INDO did not influence hyperglycemia when the 2 drugs were administered simultaneously (Figure 4 B). Administration of 2 mg/kg INDO to a diabetic animal does not de-

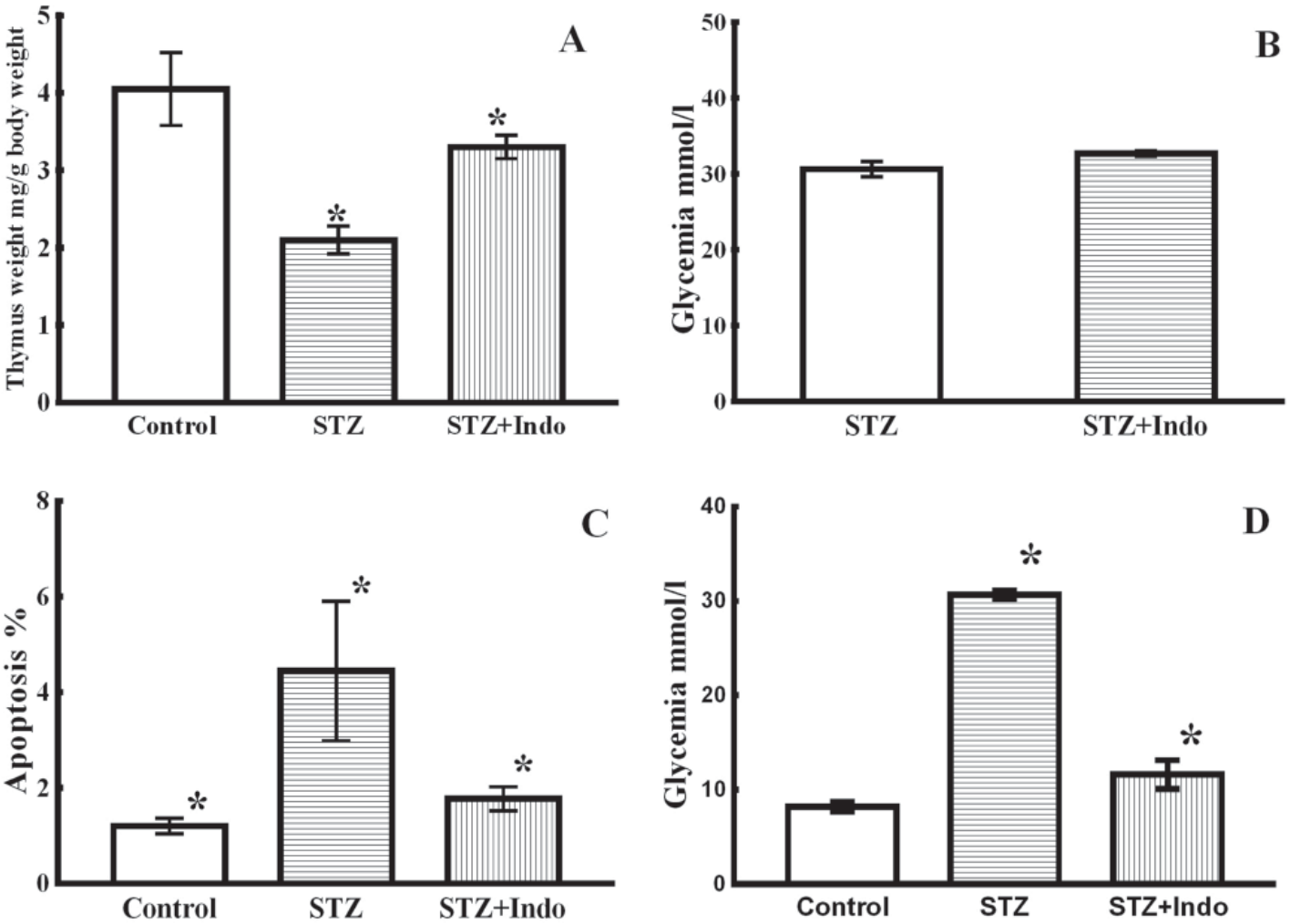

Figure 4. (A) Thymus weight at baseline and after 24 h in streptozotocin- and streptozotocin+indomethacin-treated mice that received both drugs at the same time. (B) Glycemia at 24 h in streptozotocin- and streptozotocin+indomethacin-treated animals. (C) Rate of apoptosis in the thymus of animals treated with streptozotocin- and streptozotocin+indomethacin; both drugs were given at the same time. (D) Glycemia at 24 h in streptozotocin- and streptozotocin+indomethacin-treated animals in which indomethacin was given 4 h prior to streptozotocin. *, $P < 0.05$.

crease glycemia (data not shown).

To address the possibility of insufficient inhibition of cyclooxygenase 2 at the time of STZ injection, INDO was given 4 h before STZ and thymus weight and glycemia measured 24 h later. Pretreatment with INDO inhibited hyperglycemia, which dropped significantly ($P < 0.05$) but remained above the normal range (Figure 4 D). Pretreatment with INDO also inhibited thymic involution but was no more efficient than INDO given simultaneously with STZ (data not shown).

Corticosterone is elevated in diabetic mice. Induction of diabetes in mice is accompanied by marked increases in serum corticosterone, which is the main glucocorticoid in rodents.[2,23] Its serum levels were significantly elevated at 24 h ($P < 0.05$) in hyperglycemic versus control mice (Figure 5). At 48 h corticosterone was even higher, approaching a 3-fold increase over control levels. Administration of INDO significantly reduced the corticosterone levels ($P < 0.05$), both at 24 h and 48 h (Figure 5).

Discussion

In recent years a growing body of evidence suggests a link among obesity, inflammation, and diabetes in humans.[6,8] It is now clear that obesity is associated with mild chronic inflammation and leads to insulin resistance that will ultimately result in type 2 diabetes.[8] More than 10 y ago it was discovered that the proinflammatory cytokine TNFα is overexpressed in the adipose tissue of rodent models of obesity and induces insulin resistance.[8] Later, the same overexpression was found to occur in the adipose tissue of obese humans.[6] Other inflammatory markers like C reactive protein, interleukins 1 and 6, the cyclooxygenase 2-derived prostaglandin E2, and other mediators[1,21] have been found to be associated with diabetes.

New data shed light on the intracellular signaling pathways activated by the inflammatory reactions and how they interfere with insulin signaling. Inflammatory signals lead to insulin resistance by inhibiting the signaling downstream from the insulin receptor. Exposure of cells to TNFα leads to inhibitory phosphorylation of the insulin receptor and inhibition of downstream signaling and thus insulin action.[7] In addition, the hyperlipidemia commonly associated with obesity causes insulin resistance in peripheral tissues and induces the expression of cyclooxygenase 2.[12] More-

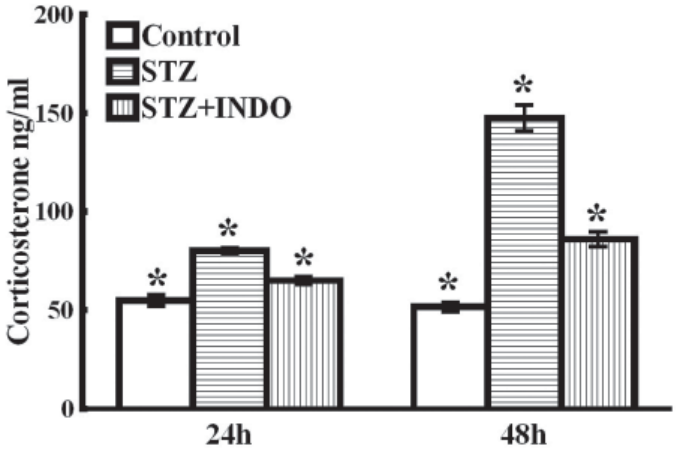

Figure 5. Corticosterone levels at 24 and 48 h in streptozotocin- and streptozotocin+indomethacin-treated mice. *, $P < 0.05$.

over, hyperglycemia induces oxidative and inflammatory stress,[17] whereas insulin inhibits oxidative stress and the activation of inflammatory mechanisms.[4] Chronic hyperglycemia in STZ-treated rats leads to an increase in blood glucocorticoids, and these stress hormones can induce thymocytes apoptosis and thymic involution, among other effects.[2,23]

The experimental diabetes induced by a single, massive dose of STZ leads quickly to persistent hyperglycemia and rapid thymic involution. Few studies have attempted to explain thymic involution in this model. Although STZ was found to be toxic to primary lymphoid tissues,[26] in our experiments, exposure of rats to a diabetogenic dose of STZ for 18 h (before the onset of hyperglycemia) did not cause thymic involution. In addition, thymocytes exposed in culture to high STZ concentrations (100 µM and 1 mM) for 6 h or 24 h did not develop apoptosis. Given this evidence and its low half-life in serum,[22] STZ likely is not directly responsible for the thymic involution in experimental diabetes.

High glucose causes oxidative stress in cultured neurons, leading to mitochondrial dysfunction and eventually their apoptosis.[20] Hyperglycemia was considered to cause thymic involution in diabetes by inhibiting Ca^{2+} ATPase, causing increased intracellular calcium levels and thymocyte apoptosis.[11] In our study, exposure to high glucose did not harm the thymocytes.

In agreement with previous reports,[2,23] in our study corticosterone (the main glucocorticoid in mice) began to increase after induction of diabetes, reaching a 3-fold increase in the hyperglycemic animals over the controls at 48 h. This finding is consistent with the proinflammatory and stress-inducing activity of hyperglycemia. Elevated corticosterone might offer an explanation for the rapid involution of the thymus in experimental diabetes, given that thymocytes are well known for their sensitivity to glucocorticoids.[5,16] The inhibition of cyclooxygenase 2 by administration of INDO may lead to systemic reduction in corticosterone levels, potentially explaining the slowdown in thymic involution.

Cyclooxygenase 2-derived metabolites interfere with the dexamethasone-induced thymocytes apoptosis and thymic involution in vivo.[16] INDO administered concomitantly with STZ inhibited thymic involution (Figure 4 A) but failed to prevent or reduce hyperglycemia (Figure 4 B). INDO inhibits thymic apoptosis, thus perhaps explains the observed differences in thymus weights (Figure 4 C). The percentage of animals that developed hyperglycemia was the same in the STZ- and STZ+INDO-treated animals when both drugs were injected simultaneously, suggesting that the 2 drugs do not interact chemically. The fact that thymic involution in STZ-treated animals is inhibited by the INDO whereas hyperglycemia is not affected suggests that hyperglycemia per se is not responsible for thymic involution and rather that other factors triggered by hyperglycemia affect the thymus. When INDO is administered 4 h before STZ, thymic involution is inhibited as expected while hyperglycemia drops significantly (but does not return to normoglycemia), pointing to the involvement of cyclooxygenase 2-derived prostanoids in STZ-mediated β-cell destruction[25] (Figure 4 D). However, giving INDO to an already diabetic animal did not influence glycemia (data not shown), suggesting that indeed, inhibition of cyclooxygenase 2 protects β cells from STZ-mediated destruction.

The results of the present study show that thymic involution, a common feature of experimental diabetes, is unlikely to be caused by STZ or hyperglycemia. Rather, the cyclooxygenase 2 pathway seems to be involved due to the protective effect that its inhibitor, indomethacin, exerts against thymic involution.

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