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Rat Pancreatic Islet and RINm5F Cell Responses to Epiandrosterone, Dehydroepiandrosterone and Interleukin-1β

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ABSTRACT. Epiandrosterone (EA), dehydroepiandrosterone (DHEA), and their sulfate (-S) and acetate (-A) conjugates were investigated for effects on isolated pancreatic islets and RINm5F insulinoma cells. Interleukin-1β (IL-1β) inhibited glucose-stimulated insulin release in cultured islets, but the presence of EA, EA-A, and to a lesser extent EA-S, preserved the secretory response. IL-1β also increased islet nitrite production, which was antagonized by EA and EA-A, but not by EA-S. EA, EA-A, DHEA, and DHEA-A, but not EA-S and DHEA-S inhibited glucose-stimulated insulin release from islets. This response may be related to the inhibition of glucose transport by EA, EA-A, DHEA, DHEA-A, and DHEA-S, as observed in RINm5F cells. EA, EA-A, DHEA, and DHEA-A also inhibited glucose metabolism in RINm5F cells, whereas EA-S and DHEA-S had no effect. EA, EA-A, DHEA, and DHEA-A, but not the sulfate conjugates, also inhibited RINm5F cell IL-1β-induced nitric oxide synthase (iNOS) activity. IL-1β also increased cytosolic Cu/Zn-superoxide dismutase (SOD) and mitochondrial Mn-SOD in RINm5F cells. EA inhibited RINm5F cell Cu/Zn-SOD in the presence and absence of IL-1β, whereas EA-S increased basal enzyme activity and did not affect the IL-1β response. EA did not affect basal Mn-SOD activity and inhibited IL-1β-stimulated activity, whereas EA-S was without effect. IL-1β had no effect on catalase activity in RINm5F cells, whereas EA, EA-A, and DHEA-A inhibited catalase activity. Thus, EA and DHEA and their acetate congeners protected the β-cell from the inhibitory effects of IL-1β, and inhibited glucose transport and oxidation, and inducible nitricoxide synthase expression. EA and DHEA also had profound effects on Cu/Zn-SOD, which may alter the toxic effects of hydrogen peroxide generation in β-cells. BIOCHEM PHARMACOL **55**;9:1453–1464, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. islet of Langerhans; 17-ketosteroids; superoxide dismutase; catalase; glucose transport; nitric oxide synthase; beta-cell; insulinoma cell

DHEA†, an androgenic 17-ketosteroid derived from pregnenolone, is an intermediate in the biosynthesis of testosterone and estrogens. DHEA preserved β-cell structure and function when fed to genetically diabetic or obese mice prior to the onset of diabetes, and protected islet β-cells from the effects of streptozotocin-induced diabetes [1, 2]. EA is a weakly androgenic 17-ketosteroid metabolite of testosterone, is synthesized primarily in the liver, and has been demonstrated to have cellular actions similar to DHEA [3, 4]. The 17-ketosteroids circulate, in part, as the sulfated conjugates. EA and DHEA affect glucose metabolism [4, 5], suppress superoxide production [6], increase catalase activity [4], and protect cells from free radical toxicity [7]. DHEA may also increase transforming growth factor-α mRNA [8], and peroxisome proliferation in cells [9]. Previously, EA was reported to markedly inhibit glucose oxidation and utilization and the activity of the hexose monophosphate (pentose) shunt in isolated rat pancreatic islets [5]. Moreover, those effects were rapid and reversible. The effects of EA also antagonized the effects of IL-1β on pancreatic β-cells and preserved the insulin responsivity to glucose in those cells.

IL-1β induces iNOS in β-cells, increases nitric oxide production, and reduces insulin secretory responses [10–13]. The effects of IL-1β on the insulin-secreting β-cell include the inhibition of mitochondrial enzymes [14], as well as inhibition of total glucose oxidation and utilization [5]. In addition, IL-1β induces mitochondrial Mn-SOD activity in insulinoma RINm5F cells [15]. The mechanisms responsible for the protective effects of EA, and to a lesser extent DHEA, against IL-1β effects in β-cells, or for their effects on glucose metabolism, are not known. The observation that these steroids affected glucose metabolism in general, as well as the activity of the pentose shunt and the activity of glucose-6-phosphate dehydrogenase [4, 5], sug-

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† *Abbreviations:* DHEA, dehydroepiandrosterone; DHEA-A, DHEA 3-acetate; DHEA-S, DHEA 3-sulfate; 2-DOG, 2-deoxyglucose; EA, epiandrosterone; EA-A, EA 3-acetate; EA-S, EA 3-sulfate; IL-1β, interleukin-1β; KRBH, Krebs-Ringer bicarbonate HEPES; iNOS, inducible nitric oxide synthase; and SOD, superoxide dismutase.

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gested that they had direct and rapid effects on processes directly related to glucose utilization. Those studies also suggested that an effect of EA on metabolism might affect iNOS induction. In the present study, EA, DHEA and their acetate and sulfate congeners were investigated and compared for effects on glucose uptake and metabolism in β-cells, the activity of iNOS, catalase and SOD, and for protective effects against IL-1β-induced inhibitory responses.

MATERIALS AND METHODS

Materials

D-[U-^{14}C]Glucose (250–360 mCi/mmol), D-[1-^{14}C]glucose (50–60 mCi/mmol), D-[6-^{14}C]glucose (50–60 mCi/mmol), D-[5-^{3}H]glucose (10–20 Ci/mmol), and L-[U-^{14}C]arginine (200–300 mCi/mmol) were obtained from American Radiolabeled Chemicals. [^{125}I]Insulin (porcine) was from DuPont/NEN. EA (5α-androstan-3β-ol-17-one), EA-A (3β-acetoxy-5α-androstan-17-one), EA-S (5α-androstan-3β-ol-17-one sulfate, sodium salt), DHEA (5-androsten-3β-ol-17-one), DHEA-A (DHEA 3-acetate), DHEA-S (DHEA 3-sulfate, sodium salt), NADP, fatty acid-free BSA (fraction V), xanthine, cytochrome *c* (type III from horse heart), xanthine oxidase (grade I from buttermilk), hydrogen peroxide, Triton X-100, SOD, catalase, and RPMI-1640 were from the Sigma Chemical Co. Fetal bovine serum was from Atlanta Biologicals. CMRL-1066, glutamine, and antibiotics were from Life Technologies. rhIL-1β was from R&D Systems. Dowex AG 50W-X8 (sodium form, 100–200 mesh) was from Bio-Rad Laboratories. Rat insulin for radioimmunoassay standard was a gift from the Eli Lilly Co. Collagenase (type P) was from Boehringer Mannheim. All other chemicals were reagent grade. RINm5F cells were a gift from Dr. C. Wollheim.

Tissue Isolation/Culture

Islets were isolated from excised pancreata of male Sprague–Dawley rats (250 g) using collagenase digestion, and cultured for 18 hr in CMRL-1066 containing 5.5 mM of glucose, 9% fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 μg/mL) at 5% CO_2-95% air, 35°, as described previously [16]. All animal procedures were approved by the institutional Laboratory Animal Care and Use Committee. RINm5F cells (0.4×10^6 cells/well of a 24-well plate) were cultured in RPMI-1640 medium containing 11 mM of glucose and the other additions listed above for 4 days prior to the addition of the experimental drugs. EA and DHEA were solubilized in DMSO and added to islets or RINm5F cells 2 hr prior to the addition of IL-1β (1 ng/mL), and then islets/cells were cultured for an additional 18 hr. Control islets/cells received DMSO in a concentration equivalent to the test conditions of 1% or less. Viability of the cells, as determined by trypan blue exclusion, was 95% or greater following an 18-hr culture with DMSO, EA (100 μM), or DHEA (100 μM).

Glucose Oxidation

Prior to determination of glucose oxidation, islets or RINm5F cells were washed twice with KRBH buffer (pH 7.4) containing 16 mM of HEPES, 0.01% BSA for islets or 0.1% BSA for cells, a glucose concentration equivalent to that of the original culture medium, and the presence or absence of drugs also present in the original culture medium (except for IL-1β, which was removed during the washes). The islets or cells were preincubated subsequently for 90 min at 37°, in KRBH buffer such that glucose and all drug treatments (except IL-1β) were kept constant throughout culture and preincubation. In certain experiments, the steroids were omitted during the washout and preincubation steps. In other experiments (acute addition), the islets were cultured for 18 hr in CMRL-1066 medium, washed and preincubated in KRBH buffer at 5.5 mM of glucose, and then glucose oxidation was determined in the presence and absence of the steroids.

Total glucose oxidation was determined as D-[U-^{14}C]glucose oxidation to $^{14}CO_2$; glucose oxidation by the hexose-monophosphate (pentose) shunt, glycolysis, and citric acid cycle was determined as D-[1-^{14}C]glucose and D-[6-^{14}C]glucose oxidation to $^{14}CO_2$, as described previously [17–19]. Briefly, D-[U-^{14}C]glucose (0.5 to 1 μCi), or D-[1-^{14}C]glucose (0.5 to 1 μCi), or D-[6-^{14}C]glucose (0.5 to 1 μCi) was added to islets (100 μL final volume) or RINm5F cells (200 μL final volume) in KRBH buffer containing 17 mM glucose. The concentrations of all drugs (except IL-1β) were kept constant during culture, washing, preincubation, and determination of glucose oxidation. In certain experiments, the steroids were omitted during the washing, preincubation, and metabolic assay. Islets (30–50 per sample) or RINm5F cells (0.5×10^6 cells per sample) were incubated in conical plastic microfuge tubes (0.7 mL) placed inside glass shell vials (12 × 44 mm), and gassed with 95% O_2:5% CO_2 before capping with a rubber stopper. The islets/cells were incubated for 90 min at 37° in a shaking water bath. At the end of the incubation, a strip of filter paper placed between the inner tube and outer vial was saturated with NaOH (1 N; 50 μL), and HCl (1 N; 20 μL) was added to the cell tube by injection through the rubber stopper. The tubes were allowed to equilibrate for 80 min at room temperature with gentle rocking. Then the tube containing the cells was removed from the vial and microfuged, and an aliquot of the incubation medium was removed to determine total counts. The cell pellet was washed twice with BSA-free Krebs-Ringer salt solution, and protein in the pellet was determined [20]. The paper strip was placed in a scintillation vial with 10 mL of scintillant and 5 drops of glacial acetic acid, and radioactivity in $^{14}CO_2$ was determined by scintillation spectrometry. Total glucose oxidation was determined as nanomoles glucose oxidized to CO_2 based upon the specific activity of the labeled D-[U-^{14}C]glucose (17 mM). Glucose oxidation of D-[1-^{14}C]glucose and D-[6-^{14}C]glucose was expressed as actual yield (cpm $^{14}CO_2$ recovered/90 min/μg of protein)

and specific yield (cpm $^{14}CO_2$ recovered divided by the total cpm added to the islets/cells and expressed per μg of protein). The contribution of the pentose shunt (P) to glucose metabolism was calculated by comparison of the mean values for islet D-[1-^{14}C]glucose and D-[6-^{14}C]glucose specific yields (unpaired), and in RINm5F cells the paired values for each form of labeled glucose, as described previously [21, 22].

Glucose Utilization

Islet utilization of D-[5-^{3}H]glucose was determined essentially as described previously, by quantitating the conversion of D-[5-^{3}H]glucose to ^3H_2O [23]. Islets or RINm5F cells were incubated in 100 or 200 μL of KRBH buffer, respectively, containing D-[5-^{3}H]glucose (17 mM; 1 μCi per sample) for 90 min, at 37°. Then the tubes were microfuged, an aliquot of the medium was removed for determination of glucose specific activity, and a second aliquot was put in a glass vial that was placed inside a plastic scintillation vial containing 0.5 mL of water, and which was then capped. Following an overnight incubation at 37°, the inner vial was removed, and the ^3H_2O that had equilibrated to the outer vial overnight was determined by liquid scintillation counting. ^3H_2O was used as a standard to determine the percent recovery during equilibration. All sample values were corrected to 100% recovery, and blank values (reaction mixture in the absence of islets or cells) were subtracted. Tissue washed in BSA-free Krebs-Ringer salts was used for protein determination as described above.

Nitric Oxide Synthase Activity

RINm5F cells were cultured for 18 hr (as described above) in the absence or presence of IL-1β and other agents as indicated in the text. Then the cells were harvested with trypsin/EDTA, washed in phosphate-buffered saline, and homogenized by sonication in ice-cold buffer containing 250 mM of Tris-HCl, pH 7.4, 10 mM of EDTA, 10 mM of EGTA, and 0.1% Triton X-100. Protein levels were determined on the homogenates, and equal amounts of protein were added to reaction tubes containing 25 mM of Tris-HCl, 3 μM of tetrahydrobiopterin, 1 μM of FAD, 1 mM of NADPH, 10 μM of L-[U-^{14}C]arginine, and 0.6 mM of $CaCl_2$, in a total reaction volume of 50 μL. The reaction was incubated at 37° in a shaking water bath for 20 min, and stopped with 450 μL of a solution containing 50 mM of HEPES and 5 mM of EDTA, pH 5.5. Columns of Dowex resin were prepared with stopping buffer, the samples were applied, and [^{14}C]citrulline was eluted with 3.5 mL of deionized water. The radioactivity in the eluate was counted by liquid scintillation spectrometry. Blank values from samples lacking homogenate and containing homogenization buffer only in the reaction tubes were subtracted from experimental cell sample values.

Insulin Release

Freshly isolated islets (10 islets per sample) were preincubated for 30 min in KRBH buffer containing 5.5 mM of glucose, at 37° under an atmosphere of 95% O_2:5% CO_2 in a shaking water bath. Following preincubation, the islets were incubated for an additional 1 hr in fresh KRBH buffer and the presence of agents indicated in the text. Islets that had been cultured for 18 hr were washed twice with KRBH buffer containing 5.5 mM of glucose, and preincubated for 1 hr in the KRBH buffer. Then, the KRBH buffer was replaced with fresh buffer with or without other agents indicated in the text, and the islets were incubated for an additional 1 hr. In certain experiments, investigational agents were maintained at a constant concentration throughout culture, preincubation, and the insulin release assay. Aliquots of incubation medium were removed at time zero and following the 1-hr incubation for assay of insulin by radioimmunoassay using rat insulin standards. Insulin release was expressed as insulin released after 1 hr minus zero time insulin levels. There were no significant differences between zero time insulin levels among the various islet treatments. Insulin release into CMRL-1066 medium was also determined for islets cultured 18 hr in the presence and absence of pharmacologic agents.

Nitrite Measurements

Nitric oxide synthesis was estimated by the accumulation of nitrite and nitrate in islet incubations (35–40 islets per 100 μL total incubation volume) to determine the total pool of nitric oxide produced, as described previously [5, 13, 24]. Culture medium was assayed by the addition of Greiss reagent for nitrite (100 μL sample), or nitrate plus nitrite (40 μL sample) following the nitrate reductase-generated conversion of nitrate to nitrite. Nitrite was determined at an absorbance of 540 nm using a Bio-Tek EL-311 multiscan plate reader. Data are expressed as nitrite produced per 40 islets.

Glucose Transport

RINm5F cells were divided into aliquots such that there were 6–7 × 10^6 cells/mL of RPMI-1640. Then the cells were incubated at 37° under an atmosphere of O_2/CO_2 (95:5), in the absence (control) or presence of the agents indicated in the text, for 80 min. Steroids were solubilized in DMSO, and an equal volume of the vehicle was added to the control cells (≤ 1.0%). Following the incubation, the cells were washed in KRB buffer containing 2-DOG (1 mM) and the agents originally present during the incubation, and resuspended at a concentration of 1 × 10^6 cells/0.01 mL KRB buffer containing 2-DOG (1 mM). Glucose transport per 3.5 × 10^6 cells was determined in a total volume of 175 μL containing 0.35 μCi of [U-^{14}C]2-DOG (1 mM), 0.5 μCi [^{3}H]mannitol, and the drug/vehicle additions as indicated in the text. Aliquots (50 μL) were

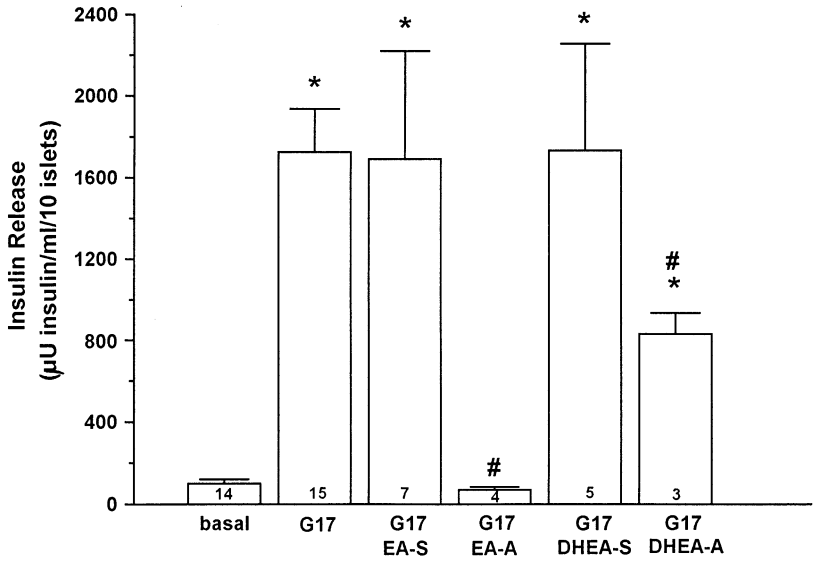

FIG. 1. Effects of EA and DHEA conjugates on insulin release in freshly isolated islets. Islets were incubated in KRBH buffer in the presence of 5.5 mM of glucose (basal) or 17 mM of glucose (G17) and EA-S (100 μM), EA-A (100 μM), DHEA-S (100 μM), or DHEA-A (100 μM), as indicated, for 60 min. Values are the means ± SEM for the number of determinations indicated at the base of each bar. *$P < 0.001$ vs basal; and #$P < 0.05$ vs G17, as determined by one-way ANOVA and the Student/Newman–Keuls multiple comparison test.

removed after 1, 2, and 4 min following the start of the reaction by the addition of cells. The aliquots were added to 1 mL of ice-cold stop solution of KRB buffer containing: 2-DOG (1 mM), phloretin (300 μM), $HgCl_2$ (80 μM), and KI (50 μM). The stopped samples were microfuged for 1 min at 14,000 rpm. An aliquot of the supernatant was removed for determination of total counts per minute and specific activities of the radiolabels, and the remaining supernatant was discarded. The cells were washed with 0.6 mL of ice-cold stop solution and microfuged for 30 sec, and the supernatant was discarded. The tip of the microfuge tube containing the cell pellet was cut off and placed in a scintillation vial containing 1 mL of sodium dodecyl sulfate (1.0%), and incubated at 37° for 30 min. Then the samples were counted by liquid scintillation spectrometry for dual label quantitation. [^{3}H]Mannitol was included as an extracellular space marker, and the 2-DOG trapped in the extracellular space was subtracted from the total 2-DOG in the samples to determine intracellular glucose uptake. Zero time values were determined by the addition of cells to stop solution prior to the addition of radiolabel, and were subtracted from the 2-DOG transport values at subsequent times of incubation.

SOD and Catalase Analyses

For determination of SOD activity, RINm5F cells were cultured for 18 hr in RPMI-1640 medium in the presence or absence of agents listed in the text, washed in phosphate-buffered saline, pH 7.4, to remove serum and other agents, and then were homogenized by sonication in potassium phosphate buffer (0.01 M) containing EDTA (1 mM) and Triton X-100 (0.2%), pH 7.8. The homogenate was microfuged for 2 min at 14,000 rpm, and the supernatant was assayed for SOD and for protein. Xanthine (0.75 mM) was dissolved in boiling buffer. Cytochrome *c* (4 mg/mL) and KCN (2 mg/mL) were dissolved in buffer. Xanthine oxidase was diluted (10 μl/mL) in buffer. SOD activity in the homogenate was determined in buffer containing cytochrome *c* (0.013 mM), xanthine (0.05 mM), xanthine oxidase (adjusted to give a rate of change in O.D. near 0.025/min in the absence of homogenate), and the absence or presence of KCN (0.3 mM). Cytosolic copper, zinc-bound (Cu/Zn)-SOD activity was determined as the difference between total SOD activity and the activity in the presence of KCN (mitochondrial Mn-SOD). Changes in O.D. at 550 nm were plotted, and the rate was recorded using a Beckman DU640B spectrophotometer. Cell samples were standardized using commercially available SOD.

For determination of catalase activity, RINm5F cells were homogenized in phosphate-buffered saline containing 0.2% Triton X-100, and microfuged for 2 min. The supernatant was used to assay catalase and for protein determination. Catalase activity in homogenate was determined in an assay mixture of sodium phosphate buffer (50 mM), pH 7.0, containing 0.2% hydrogen peroxide. The rate of change in O.D. at 240 nm was determined using a Beckman DU640B spectrophotometer. Cell samples were standardized using commercially available catalase.

Statistical Analysis

The data are presented as means ± SEM and were analyzed by Student's *t*-test, or one-way ANOVA with the Student/Newman–Keuls multiple comparison test; $P < 0.05$ was accepted as significant.

RESULTS

Insulin Release

EA and DHEA at 100 μM have been reported previously to completely inhibit glucose-stimulated insulin release in isolated rat pancreatic islets as long as the steroid was present continuously during the incubation [5]. As shown

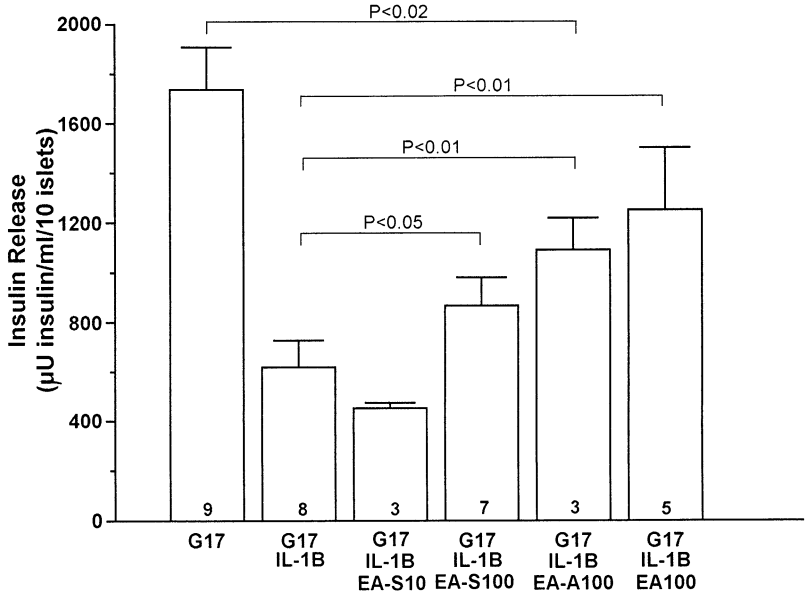

FIG. 2. Insulin release from cultured islets. Islets were cultured for 18 hr in CMRL-1066 in the absence (control) or presence of IL-1β (IL-1B; 1 ng/mL) and EA (100 μM), EA-S (10 and 100 μM), or EA-A (100 μM). Insulin release was determined in washed islets (10 per sample) in KRBH buffer in the absence of EA-S, EA-A, and IL-1β, and the presence of 17 mM of glucose (G17). Values are the means ± SEM for the number of independent determinations indicated at the base of each bar. Significant differences were determined by one-way ANOVA and the Student/Newman–Keuls multiple comparison test.

in Fig. 1, EA-A (100 μM) also markedly inhibited glucose (17 mM)-stimulated insulin release from freshly isolated rat islets, such that insulin release was not increased above basal levels. DHEA-A (100 μM) also significantly reduced glucose-stimulated insulin release ($P < 0.05$) by about 50% (Fig. 1). In contrast, neither EA-S nor DHEA-S had any effect on glucose (17 mM)-stimulated insulin release (Fig. 1), or on glucose (8.5 mM)-stimulated insulin release (data not shown). Basal insulin release from freshly isolated islets in the presence of EA-A (100 μM) (147 ± 61 μunits of insulin/mL/10 islets), EA-S (100 μM) (223 ± 49 μunits of insulin/mL/10 islets), or DHEA-A (100 μM) (150 ± 18 μunits of insulin/mL/10 islets) was not significantly different ($P > 0.05$; one-way ANOVA) from control (123 ± 29 μunits of insulin/mL/10 islets); however, DHEA-S (100 μM) (332 ± 28 μ units of insulin/mL/10 islets) slightly increased ($P < 0.02$) basal insulin release.

Previous studies have also demonstrated that culture of islets with IL-1β (1 ng/mL) for 18 hr inhibits insulin release, and that the presence of EA (100 μM) or DHEA (100 μM) during islet culture with IL-1β fully protects glucose-stimulated insulin secretion in washed islets [5]. Similarly, in the present studies, islet culture with IL-1β inhibited ($P < 0.001$) insulin release in washed islets (Fig. 2). Islets cultured with IL-1β and EA-S (100 μM), and then washed, showed a significant although modest enhancement of the glucose-stimulated insulin release response compared with islets treated with IL-1β alone (Fig. 2). EA-S (10 μM) did not affect insulin release (Fig. 2). Culture with IL-1β and DHEA-S (100 μM) did not affect insulin release significantly compared with islets treated with the cytokine alone (data not shown). Islets cultured with IL-1β and EA (100 μM), and then washed, were protected from the inhibitory effects of IL-1β, as reported previously [5] (Fig. 2). Similarly, washed islets treated with IL-1β and EA-A (100 μM) also showed significant protection of the insulin release response to glucose (Fig. 2). During 18 hr of culture, as reported previously for EA and DHEA [5], neither EA-S (100 μM) (23 ± 7 μunits of insulin/mL/islet), EA-A (100 μM) (15 ± 7 μunits of insulin/mL/islet), nor DHEA-A (100 μM) (8 ± 1 μunits of insulin/mL/islet) significantly changed ($P > 0.05$) insulin release compared with control islet values (34 ± 13 μunits/mL/islet).

Nitrite Production

Islets were cultured for 18 hr in the presence of IL-1β and the absence or presence of EA-A or EA-S in order to determine the effects of the steroids on nitrite production as a result of iNOS induction in response to the cytokine. IL-1β significantly increased nitrite levels to more than four times control levels (Fig. 3). The presence of EA-A (100 μM) with IL-1β during culture significantly reduced islet nitrite production in response to the cytokine, although levels remained higher than basal values (Fig. 3). However, a similar concentration of EA-S had no effect on islet nitrite production in response to IL-1β (Fig. 3). The steroids had no effect on control levels of nitrite (Fig. 3).

iNOS Activity

The activity of iNOS was determined in control RINm5F cells and cells treated with IL-1β in the absence and presence of EA and DHEA and their acetate and sulfate conjugates. In this assay, the recovery of [^{14}C]citrulline is proportional to the production of nitric oxide from L-[^{14}C]arginine. Culture with IL-1β for 18 hr markedly increased the expression of iNOS in RINm5F cells (Fig. 4). The presence of EA and DHEA profoundly inhibited the activity of iNOS, and EA-A and DHEA-A were nearly as effective at inhibiting the expression of this enzyme (Fig.

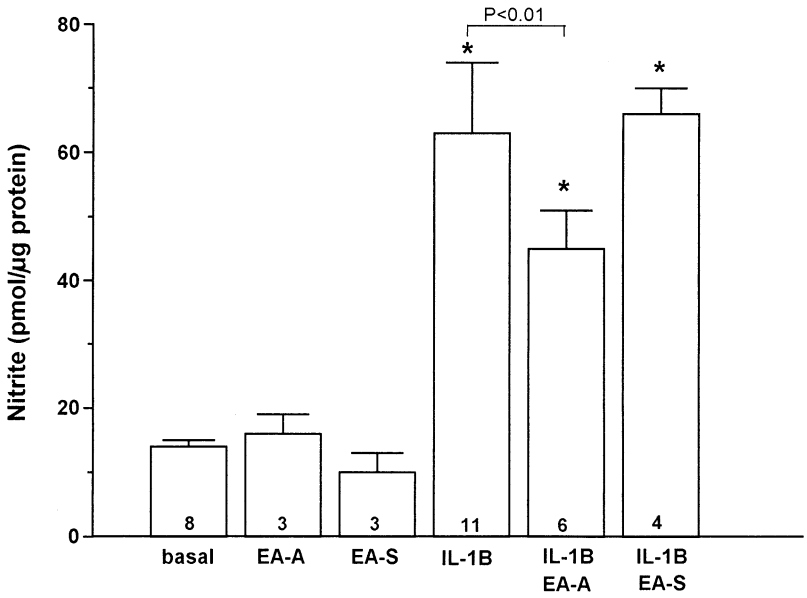

FIG. 3. Nitrite accumulation in islet cultures. Islets were cultured in CMRL-1066 for 18 hr in the absence (basal) or presence of IL-1β (IL-1B) (1 ng/mL) and EA-A (100 μM) or EA-S (100 μM), as indicated. Values are the means ± SEM for combined nitrate/nitrite accumulation for the number of determinations shown at the base of each bar. *$P < 0.01$ vs basal, as determined by one-way ANOVA and the Student/Newman–Keuls multiple comparison test.

4). EA-S and DHEA-S did not affect RINm5F iNOS expression significantly (Fig. 4).

Glucose Transport

The effects of the 17-ketosteroids on glucose transport were investigated. [U-^{14}C]2-DOG uptake in RINm5F cells was used to quantitate glucose transport, since 2-DOG is phosphorylated but not metabolized in β-cells. Glucose transport was linear for at least 4 min (data not shown). EA and DHEA evoked concentration-dependent reductions in glucose transport in RINm5F cells (Table 1). EA-A, DHEA-A, and DHEA-S also significantly reduced glucose transport to different degrees, although EA-S did not affect glucose transport (Table 1). EA (100 μM) reduced glucose transport to a greater extent ($P < 0.02$) than did similar concentrations of EA-A, DHEA-A, or DHEA-S.

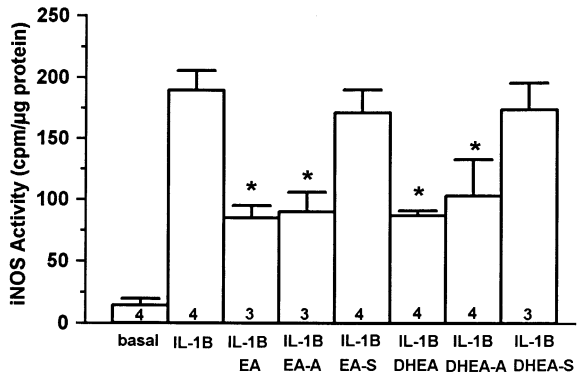

FIG. 4. iNOS expression in RINm5F cells. RINm5F cells were cultured overnight in the absence (basal) or presence of IL-1β (IL-1B; 0.1 ng/mL) and 100 μM each EA, EA-A, EA-S, DHEA, DHEA-A, or DHEA-S, as indicated. iNOS activity in cell homogenates was determined by the metabolism of [U-^{14}C]arginine to [^{14}C]citrulline. Values are the means ± SEM for the number of independent determinations shown at the base of each bar. Each determination was performed in triplicate. IL-1β values were significantly different ($P < 0.001$) from basal; an asterisk (*) indicates $P < 0.02$ vs IL-1β, as determined by two-way ANOVA and the Student/Newman–Keuls multiple comparison test.

Glucose Metabolism

A previous study demonstrated that EA (100 μM) and DHEA (100 μM) significantly inhibited islet and RINm5F cell glucose oxidation by approximately 50% [5]. In the present studies, RINm5F cells were incubated in the presence or absence of EA-A, EA-S, DHEA-A, or DHEA-S, with [U-^{14}C]glucose in order to determine effects of the steroid congeners on glucose oxidation. EA-A and DHEA-A markedly inhibited glucose oxidation in RINm5F cells (Table 2). EA-S and DHEA-S, in contrast, did not affect glucose oxidation significantly (Table 2). Similar

TABLE 1. Glucose transport in RINm5F cells

Treatment	Glucose transport (pmol 2-DOG/μg protein/min)	(N)
Control	2.2 ± 0.1	14
EA (1 μM)	2.0 ± 0.1	3
EA (10 μM)	1.6 ± 0.1*†	4
EA (100 μM)	0.3 ± 0.04*†	3
EA-A (100 μM)	0.9 ± 0.2*	3
EA-S (100 μM)	1.9 ± 0.1	4
DHEA (10 μM)	1.4 ± 0.1*	3
DHEA (100 μM)	0.9 ± 0.1*†	4
DHEA-A (100 μM)	1.2 ± 0.1*	3
DHEA-S (100 μM)	1.2 ± 0.02*	3

RINm5F cells were incubated in the presence of [U-^{14}C]2-DOG (1 mM) in the absence (control) or presence of the agents indicated. Values are the means ± SEM for the number of independent experiments indicated (*N*). Statistical differences were determined by one-way ANOVA and the Student/Newman–Keuls multiple comparison test.

*$P < 0.01$ vs control.

†$P < 0.01$ vs values for the next lowest concentration of the same drug treatment.

TABLE 2. Total glucose oxidation in RINm5F cells

Treatment group	D-[U-^{14}C]Glucose oxidation (pmol glucose/μg protein)	(N)
Control	38 ± 7	3
EA-A	7 ± 1*	3
DHEA-A	10 ± 2*	3
EA-S	39 ± 8	3
DHEA-S	32 ± 9	3

RINm5F cells were incubated in the absence (control) or presence of the EA or DHEA sulfate (-S) or acetate (-A) congeners, at 100 μM each, with [U-^{14}C]glucose. Glucose oxidation rates were determined during a 90-min incubation. Values are the means ± SEM for the number of independent experiments indicated (N).

*$P < 0.01$ compared with control values, as determined by one-way ANOVA and the Student/Newman–Keuls multiple comparison test.

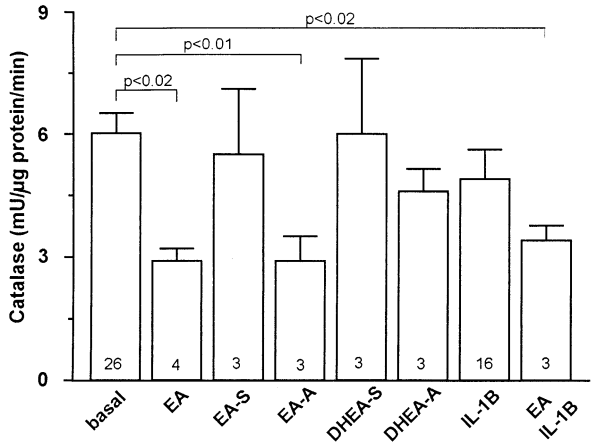

FIG. 5. Catalase activity in RINm5F cells. RINm5F cells were cultured for 18 hr in the absence (basal) or presence of IL-1β (IL-1B; 1 ng/mL) and 100 μM each: EA, EA-S, EA-A, DHEA-S, and DHEA-A, as indicated. The cells were washed and homogenized, and catalase activity was determined. Values are the means ± SEM for the number of determinations shown at the base of each bar. Significant differences were determined by one-way ANOVA and the Student/Newman–Keuls multiple comparison test.

results were obtained if the cells were cultured for 18 hr with the steroids, and then the steroids were also present during the glucose oxidation assays (data not shown).

In isolated islets, EA-S was investigated for its effects on the pentose shunt. Previous studies demonstrated that EA (100 μM) and DHEA (100 μM) inhibit the pentose shunt in islets [5]. When islets were cultured for 18 hr with EA-S, and then glucose oxidation was determined in the presence of the steroid, EA-S did not affect [1-^{14}C]glucose oxidation, but this steroid did increase the oxidation of [6-^{14}C]glucose in islets significantly (Table 3). The metabolism of [5-^{3}H]glucose, as a quantitative measure of total glucose utilization (GLU), in islets treated with EA-S was not significantly different from untreated control cells (Table 3). The contribution of the pentose shunt to glucose oxidation (P) in islets treated with EA-S was significantly lower than in control islets, and the activity of the pentose shunt (PS) was lower than in the control (Table 3). DHEA-S did not affect [1-^{14}C]glucose oxidation, but also did not affect significantly [6-^{14}C]glucose oxidation or the utilization of [5-^{3}H]glucose (Table 3).

Catalase Activity

Since isolated islets are a heterogeneous cell population of endocrine and non-endocrine cells, the insulinoma β-cell line RINm5F was studied to determine the effects of cytokine and steroids on the antioxidant enzymes catalase and SOD. RINm5F cell catalase activity was inhibited following culture with EA (100 μM) or a similar concentration of EA-A, although IL-1β and DHEA-A did not affect catalase activity significantly in these cells (Fig. 5). A combination of IL-1β and EA also reduced catalase activity. Neither of the sulfate conjugates of EA or DHEA affected catalase activity (Fig. 5).

TABLE 3. Glucose metabolism and pentose shunt activity in islets

Actual yield (cpm/μg protein)		Specific yield (x 10^{-5})				
[1-^{14}C]-Glucose	[6-^{14}C]-Glucose	[1-^{14}C]-Glucose	[6-^{14}C]-Glucose	P (x 10^{-6})	GLU (pmol glucose utilized/μg protein)	Pentose shunt (fmol glucose oxidized/μg protein)
			Control			
60.0 ± 2.9 (5)	27.8 ± 2.3 (5)	2.6 ± 0.1 (5)	1.3 ± 0.1 (5)	4.3 ± 0.3 (5)	240 ± 42 (4)	1.03
			EA-S			
68.1 ± 1.2 (3)	43.0 ± 1.1* (3)	2.8 ± 0.1 (3)	1.9 ± 0.1* (3)	2.8 ± 0.03* (3)	318 ± 44 (3)	0.89
			DHEA-S			
71.1 ± 8.5 (3)	30.9 ± 8.3 (3)	3.4 ± 0.6 (3)	1.5 ± 0.5 (3)	6.3 ± 0.9 (3)	297 ± 43 (3)	1.87

Islets were cultured for 18 hr in the absence (control) or presence of EA-S (100 μM) or DHEA-S (100 μM). Glucose oxidation and total glucose utilization (GLU) (90 min) were determined in the absence (control) or presence of EA-S (100 μM) or DHEA-S (100 μM), as indicated. Glucose oxidation is expressed as the actual yield (cpm $^{14}CO_2$/μg of protein) and specific yield (cpm $^{14}CO_2$ recovered divided by the total cpm per sample per μg of protein). The fraction of glucose oxidation contributed by the pentose shunt (P) for islets was calculated by comparison of the paired values for D-[1-^{14}C]glucose and D-[6-^{14}C]glucose specific yields. The amount of glucose metabolized through the pentose shunt was determined by comparison of mean P and GLU values. Values are the means ± SEM as indicated, for the number of experiments (*N*).

*$P < 0.01$ vs control values within a group, as determined by one-way ANOVA and the Student/Newman–Keuls multiple comparison test.

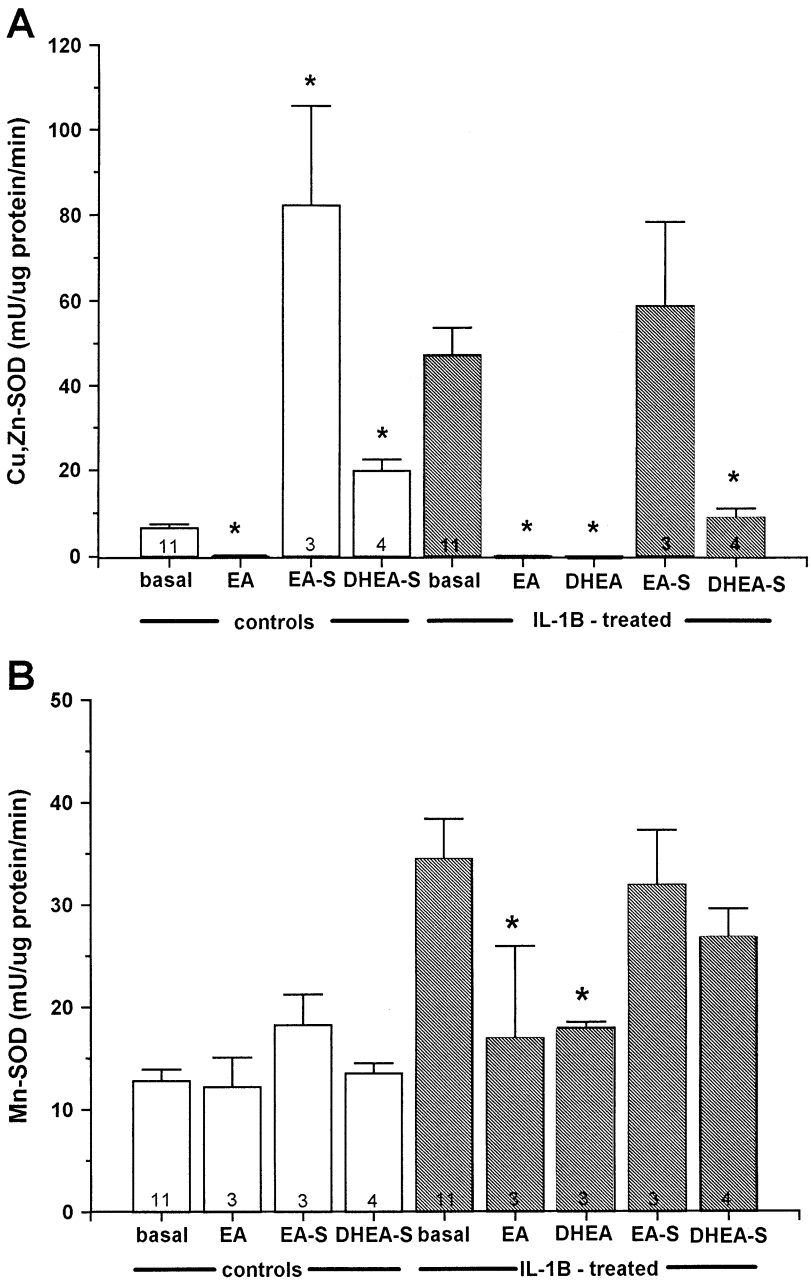

FIG. 6. Copper/zinc bound (Cu/Zn)-SOD and manganese (Mn)-SOD activities in RINm5F cells. (A) Cu/Zn-SOD activity was determined in homogenates of washed RINm5F cells that had been cultured for 18 hr in the absence (control) or presence of IL-1β (IL-1B; 1 ng/mL) and 100 μM each: EA-S or DHEA-S, as indicated. (B) Mn-SOD activity was determined as in (A). Values are the means ± SEM for the number of independent experiments shown at the base of each bar (EA, EA + IL-1β, and DHEA + IL-1β are each N = 3). Significant differences were determined by one-way ANOVA and the Student/Newman–Keuls multiple comparison test. *P < 0.05 vs basal for the respective treatment groups.

SOD Activity

Cytosolic Cu/Zn-SOD activity was four times higher in homogenates of washed RINm5F cells previously cultured with IL-1β, and seven times higher in cells cultured with EA-S (100 μM) (Fig. 6A). Culture with DHEA-S (100 μM) only slightly increased Cu/Zn-SOD activity. In sharp contrast, EA (100 μM) in the absence or presence of IL-1β reduced Cu/Zn-SOD activity to undetectable levels, and the presence of DHEA (100 μM) evoked a similar response (Fig. 6A). Culture with DHEA-S completely inhibited the stimulatory effects of IL-1β on enzyme activity (Fig. 6A). In contrast, EA-S did not affect significantly IL-1β effects on Cu/Zn-SOD activity (Fig. 6A).

Mitochondrial Mn-SOD activity in homogenates of washed RINm5F cells was increased two-and-a-half times above control values following culture of cells with IL-1β (Fig. 6B). EA (100 μM) and DHEA (100 μM) completely inhibited the stimulatory response to IL-1β (Fig. 6B). However, neither EA-S nor DHEA-S was effective in significantly reducing the response to IL-1β (Fig. 6B). EA, EA-S, and DHEA-S did not affect basal Mn-SOD activity (Fig. 6B).

DISCUSSION

Rat pancreatic β-cell insulin secretory responses are inhibited following exposure of the cells to IL-1β as illustrated in this study and reported previously [5, 25, 26]. IL-1β induces changes in (pro)insulin biosynthesis and metabolism [13, 27], DNA fragmentation and apoptosis [28–30], and iNOS

activity [31, 32]. Previous studies have shown that the deleterious effects of IL-1β on insulin release can be inhibited during treatment of β-cells with EA or DHEA [5]. These steroids were reported to have inhibitory effects on β-cell glucose oxidation and utilization, and on the production of nitric oxide in response to IL-1β. Although EA and DHEA can inhibit glucose-6-phosphate dehydrogenase and the pentose shunt [4, 5], and thereby reduce the production of NADPH (required for nitric oxide synthase activity) [33], the mechanism whereby these steroids affect total glucose utilization and protect the cell from the effects of IL-1β were not identified. It has been reported that in db/db mice, DHEA treatment results in the preservation of β-cell structure and function [1, 2], and DHEA was therapeutic to streptozotocin-treated mice that ordinarily would become diabetic. However, the cellular mechanisms mediating these responses are not known.

It was reported previously that EA and DHEA inhibit insulin release if the steroids are present during the incubation with glucose to induce secretion [5]. In the present study, EA-A and DHEA-A also inhibited insulin release in response to glucose. However, EA-S and DHEA-S did not mimic the effects of the acetate congeners, and did not have any acute effects on glucose-stimulated insulin release from isolated islets. The acetate and sulfate conjugates of EA and DHEA were studied to determine if the conjugates were more or less potent than the parent compounds. In these studies, the islet responses to the acetate conjugates of EA and DHEA closely paralleled the responses evoked by the parent steroids. The acetate form of the steroids is more water soluble, and dissociates to the active free steroid. However, the lack of islet secretory response to the sulfated steroid congeners suggests that the islets do not possess sufficient sulfatase activity to provide active steroid levels and/or that the sulfated conjugates are bound to serum proteins and are largely unavailable to the cells. *In vivo*, DHEA-S is secreted from the adrenal gland, enters the circulation, and is excreted by the kidney. However, it would appear from the present results that the sulfated form of the 17-ketosteroids would not affect secretory responses in the islet unless they were desulfated and available as the free steroid.

Since glucose oxidation and utilization were reported previously to be inhibited by EA and DHEA in islets and RINm5F cells [5], the effects of the 17-ketosteroids on glucose transport were investigated. Glucose transport in β-cells is mediated by a facilitative glucose transporter, GLUT-2 [34]. In the present study, EA and DHEA inhibited glucose uptake into RINm5F cells in a concentration-dependent manner. These results appear to explain the inhibitory effects of EA and DHEA on glucose oxidation and utilization in β-cells. The small inhibition of glucose uptake by DHEA-S was not accompanied by a reduction in glucose oxidation, probably because modest changes in glucose uptake are not sufficient to limit glucose metabolism in islets. It has been reported that glucose utilization depends upon functional glucose transporter and glucokinase [35], and that underexpression of the β-cell GLUT-2 to less than 19% of controls in Zucker diabetic fatty rats is associated with reduced glucose uptake and impaired glucose-stimulated insulin secretion [36]. In transgenic mice showing an 80% reduction in β-cell GLUT-2, there was an almost total inhibition of glucose-induced insulin secretion from isolated islets [37]. Thus, it is likely that the as much as 86% inhibition of glucose transport in the presence of 17-ketosteroids observed in the present study influenced glucose metabolism and insulin secretion. Unlike its effect on β-cells, DHEA has been reported to enhance glucose uptake in fibroblasts after 10 hr or more of culture [38]. On the other hand, glucorticoids are known to inhibit glucose transport [39], and sex steroids have been reported to inhibit other types of transport processes [40]. The different glucose transport mechanisms in various cell types may account for regulatory differences in response to the steroids. EA-A, DHEA-A, and DHEA-S also reduced glucose transport in RINm5F cells, although EA-S was without effect at the concentrations studied. Thus, the lack of an acute inhibitory effect of DHEA-S and EA-S on insulin release correlated with the little or no effect on glucose transport in β-cells.

The effects of the 17-ketosteroid congeners on β-cell glucose oxidation were also determined. Total glucose oxidation determined using D-[U-^{14}C]glucose indicated that EA-A and DHEA-A reduced metabolism in RINm5F cells, similar to the effects of EA and DHEA in a previous study [5]. The lack of an effect of EA-S on total glucose oxidation suggests that the changes in glucose transport noted in this study may be primarily responsible for the changes in total glucose oxidation observed with certain of the 17-ketosteroids. However, since EA and DHEA inhibit glucose-6-phosphate dehydrogenase [4], the rate-limiting enzyme in the pentose shunt which serves as the major source of cytsolic NADPH in mammalian cells, the effect of EA-S on glucose utilization and oxidation through the pentose shunt was investigated. EA-S significantly increased [6-^{14}C]glucose oxidation through mitochondrial pathways as compared with control islet values. When the fraction of glucose metabolized through the pentose shunt was estimated from the changes in glucose oxidation, there was a significant decrease in glucose oxidized through the pentose pathway in EA-S-treated islets. While EA and DHEA have been reported previously to reduce glucose oxidation through the pentose shunt, these steroids reduced both pentose shunt and mitochondrial contributions to glucose oxidation [5]. In contrast, EA-S changed the ratio of glucose utilization such that the mitochondrial contribution was increased markedly following an 18-hr incubation, and the contribution of the pentose shunt was reduced. The mechanism accounting for this stimulatory response is not known. The lack of an inhibitory effect of EA-S on glucose oxidation and utilization probably accounts for the lack of effect of this steroid on insulin release in response to glucose in this study, since glucose metabolism is required for insulin release [34]. Previously, the

metabolic and secretory effects of EA and DHEA were shown to occur rapidly and in parallel, and to be unrelated to changes in insulin content, total protein content, DNA content, or cell number [5].

The presence of IL-1β during the 18-hr islet culture inhibited glucose-stimulated insulin release. In addition, similar to the previously reported results with EA and DHEA [5], the presence of EA-A during culture of islets with the cytokine enhanced the insulin secretory response by about 83%, thus partially protecting the β-cells and the insulin secretory response to glucose. However, an unexpected finding, was that EA-S when present during islet 18-hr culture with IL-1β also enhanced to a modest extent (approximately 42%) insulin release in comparison with cytokine-treated control islets. Since EA-S failed to affect insulin release upon acute exposure of islets to these agents, unlike EA or EA-A, the mechanism of action of EA-S in providing a small degree of protection of the β-cell from the effects of IL-1β would appear to be different from the actions of the other 17-ketosteroids investigated, although this mechanism has yet to be elucidated.

Nitric oxide (estimated in these studies through the quantitation of nitrite levels), synthesized by iNOS in response to IL-1β, appears to be responsible for toxic effects on glucose metabolism [10, 14]. Previously, EA and DHEA were reported to inhibit nitrite formation in response to IL-1β in islets and RINm5F cells [5]. As with the parent steroid, EA-A antagonized the IL-1β effects on glucose metabolism, insulin release, and also the production of nitrite. On the other hand, EA-S, which lacked acute inhibitory effects on glucose oxidation and insulin release, also did not affect nitrite production in response to IL-1β during an 18-hr culture. However, following the 18-hr culture, EA-S protected the β-cells to a small extent from the effects of IL-1β and partially preserved glucose-stimulated insulin release. Thus, unlike the other 17-ketosteroids investigated in this study, the modest protective effects of EA-S on insulin release in the presence of the cytokine do not appear to be related to a detectable reduction in glucose metabolism or nitric oxide production. In addition, although EA-S reduced pentose shunt activity in islets, this alteration in glucose metabolism and presumably NADPH production was not sufficient to limit IL-1β-induced iNOS activity and nitrite levels. When iNOS expression in response to IL-1β was investigated, it was found that EA, DHEA, EA-A, and DHEA-A each significantly inhibited iNOS expression in response to the cytokine, whereas the sulfated forms of the steroids were not effective. Thus, the expression of iNOS appears to correlate with changes in glucose utilization in response to the active steroids, and it is likely that the metabolic state of the β-cell at least partly mediates steroid-induced changes in iNOS activity and nitric oxide production and probably accounts for protective effects against IL-1β. It is not known if EA or DHEA might modulate other processes also associated with iNOS expression, such as NF-κB activation.

Recent reports suggest that active oxygen is an important factor in the destruction of the β-cell in the development of insulin-dependent diabetes mellitus. SOD is active in removing superoxide free radical by conversion to hydrogen peroxide, and catalase is active in converting cytotoxic hydrogen peroxide to water and oxygen. Exposure of β-cells to IL-1β is associated with increased Mn-SOD mRNA, and SOD induction has been considered as a defense mechanism in cells experiencing increased oxygen free radical production [15]. In addition, SOD and catalase activities are increased in various tissues of streptozotocin-induced diabetic rats, with SOD increased in pancreas [41]. SOD also has been reported to protect islets from the diabetogenic effects of alloxan [42]. In addition, elevation of cytosolic Cu/Zn-SOD by introduction of a transgene increases the tolerance of β-cells to oxidative-stress-induced diabetogenesis [43].

In the present studies, EA and EA-A inhibited β-cell catalase activity, whereas IL-1β was without effect. On the other hand, IL-1β markedly increased Cu/Zn-SOD activity, while EA completely inhibited both basal and cytokine-stimulated Cu/Zn-SOD activity. DHEA and DHEA-S also inhibited IL-1β-induced Cu/Zn-SOD activity. Surprisingly, EA-S, and to a lesser extent DHEA-S, stimulated basal Cu/Zn-SOD activity, whereas only DHEA-S antagonized IL-1β-stimulation of this enzyme. IL-1β also increased the activity of islet Mn-SOD, and EA and DHEA were effective at completely inhibiting the activity. None of the sulfate congeners of the 17-ketosteroids affected Mn-SOD activity. Thus, it would appear that the protective effects of EA and DHEA against IL-1β-induced cytotoxicity may involve a reduction in the capacity of the β-cell to generate toxic hydrogen peroxide through SOD. Since catalase activity was not affected significantly during islet exposure to IL-1β, and EA did not affect enzyme activity in cytokine-treated cells, it is unlikely that altered catalase activity plays a role in IL-1β- or EA-induced protective effects on insulin release.

In conclusion, EA, DHEA and their acetate congeners antagonize the effects of IL-1β in pancreatic islet β-cells, perhaps due to effects on glucose transport/metabolism and iNOS activity. The inhibition of glucose oxidation by these steroids is likely mediated by the inhibition of glucose transport into the β-cell. The effects of the steroids on glucose-stimulated insulin release and nitrite production mimicked the responses of glucose oxidation, except for the small protective effect of EA-S against IL-1β-treated insulin secretory responses. The dramatic effects of EA and DHEA on Cu/Zn-SOD suggest that these steroids may also protect the cell from the toxicity associated with hydrogen peroxide generation. The sulfate congeners of EA and DHEA were largely lacking in activity, perhaps due to a low level of sulfatase activity in islet tissue. However, in certain instances, such as Cu/Zn-SOD activation, both EA-S and DHEA-S displayed a stimulatory potential in exact opposition to the inhibitory effects of the parent steroids. The mechanisms responsible for these effects are not known. Thus, EA and DHEA are likely to mediate protective

effects on pancreatic β-cells indirectly through changes in metabolism, iNOS expression, and perhaps antioxidant enzyme activities.

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