

# Inhibition of Interleukin-1 $\beta$ -Induced COX-2 and EP3 Gene Expression by Sodium Salicylate Enhances Pancreatic Islet $\beta$ -Cell Function

Phuong Oanh T. Tran, Catherine E. Gleason, and R. Paul Robertson

Previous work has suggested that functional interrelationships may exist between inhibition of insulin secretion by interleukin (IL)-1 $\beta$  and the endogenous synthesis of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) in the pancreatic islet. These studies were performed to ascertain the relative abundance of E prostaglandin (EP) receptor mRNAs in tissues that are major targets, or major degradative sites, of insulin; to identify which EP receptor type mediates PGE<sub>2</sub> inhibition of insulin secretion in pancreatic islets; and to examine possible sites of action through which sodium salicylate might affect IL-1 $\beta$ /PGE<sub>2</sub> interactions. Real-time fluorescence-based RT-PCR indicated that EP3 is the most abundant EP receptor type in islets, liver, kidney, and epididymal fat. EP3 mRNA is the least, whereas EP2 mRNA is the most, abundant type in skeletal muscle. Misoprostol, an EP3 agonist, inhibited glucose-induced insulin secretion from islets, an event that was prevented by preincubation with pertussis toxin, by decreasing cAMP. Electromobility shift assays demonstrated that sodium salicylate inhibits IL-1 $\beta$ -induced nuclear factor- $\kappa$ B (NF- $\kappa$ B) activation. Sodium salicylate also prevented IL-1 $\beta$  from inducing EP3 and cyclooxygenase (COX)-2 gene expression in islets and thereby prevented IL-1 $\beta$  from inhibiting glucose-induced insulin secretion. These findings indicate that the sites of action through which sodium salicylate inhibits these negative effects of IL-1 $\beta$  on  $\beta$ -cell function include activation of NF- $\kappa$ B as well as generation of PGE<sub>2</sub> by COX-2. *Diabetes* 51: 1772–1778, 2002

**I**nterleukin-1 $\beta$  (IL-1 $\beta$ ) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) both inhibit glucose-induced insulin secretion from the pancreatic islet (1–11). The similarity of their actions and that both are mediators of inflammation have led to a consideration of whether PGE<sub>2</sub> might mediate the effects of IL-1 $\beta$  to inhibit insulin secretion (7,12). Most recently it was demonstrated that two specific antagonists of cyclooxygenase (COX)-2 activity prevent IL-1 $\beta$ -induced inhibition of glucose-induced insulin secretion from rat islets, and that this cytokine action could be

From the Pacific Northwest Research Institute and Departments of Medicine and Pharmacology, University of Washington, Seattle, Washington.

Address correspondence and reprint requests to R. Paul Robertson, Pacific Northwest Research Institute, 720 Broadway, Seattle, WA 98122. E-mail: rpr@u.washington.edu.

Received for publication 24 August 2001 and accepted in revised form 26 February 2002.

COX, cyclooxygenase; EP, E prostaglandin; FBS, fetal bovine serum; IL, interleukin; NF- $\kappa$ B, nuclear factor- $\kappa$ B; NSAID, nonsteroidal anti-inflammatory drug; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>.

restored by the addition of exogenous PGE<sub>2</sub> in the presence of the COX-2 inhibitors (13). PGE<sub>2</sub> exerts its action on cells by interacting with one or more of its four E prostaglandin (EP) receptor types, namely, EP1, EP2, EP3, or EP4. All are coupled to signal transduction systems involving phosphoinositide hydrolysis, calcium, or adenylate cyclase activity, but only EP3 has been shown to have postreceptor activities resulting in a decrease in cAMP (14,15).

In addition to its ability to inhibit the production of prostaglandins from arachidonic acid by decreasing COX-2 activity (4,16), sodium salicylate is known to inhibit nuclear factor- $\kappa$ B (NF- $\kappa$ B) activation in nonislet tissues (17,18). The promoter regions of the COX-2 and EP receptor genes have in common a putative NF- $\kappa$ B binding site that positively regulates gene expression (19,20). Sodium salicylate has been shown to decrease IL-1 $\beta$  induction of COX-2 gene expression in human umbilical vein endothelial cells (21), but its effects on COX-2 or EP3 gene expression have not been examined in islets.

The studies described in this article were designed to address the overall hypothesis that sodium salicylate's ability to block the inhibitory effect of IL-1 $\beta$  on glucose-induced insulin secretion involves inhibition of IL-1 $\beta$ -dependent activation of NF- $\kappa$ B and COX-2 and EP3 receptor gene expression. Specifically, we sought to 1) compare the relative abundance of EP receptor mRNAs in islets, the source of insulin secretion, to those in kidney, liver, muscle, and fat, which are major sites of insulin action and metabolism; 2) ascertain whether the EP3 receptor type mediates PGE<sub>2</sub> inhibition of insulin secretion; and 3) determine whether sodium salicylate, via inhibition of NF- $\kappa$ B activation, negatively regulates COX-2 and EP3 gene expression in the islet.

## RESEARCH DESIGN AND METHODS

**Materials.** Human recombinant IL-1 $\beta$  (ED<sub>50</sub> 5–10 pg/ml, cat. no. 201-LB) was obtained from R&D Systems (Minneapolis, MN). Sodium salicylate was obtained from Sigma Chemicals (St. Louis, MO). Pertussis toxin was obtained from Cayman Chemicals (Ann Arbor, MI). NS-398, PGE<sub>2</sub>, and AH-6809 were obtained from Biomol (Plymouth Meeting, PA). SC-236 was a gift from Monsanto-Searle (St. Louis, MO).

**Pancreatic islet isolation.** Pancreata from male Wistar rats were infused with 10 ml of a 0.09% collagenase type XI (Sigma), 1% fetal bovine serum (FBS), and 2 units/ml RQ1 DNase (Promega, Madison, WI) solution in Medium 199 (Sigma). After surgical removal, the pancreata were incubated in the collagenase solution at 37°C. Undigested tissue was removed using a 500- $\mu$ m screen, and the recovered tissue was washed twice with ice-cold Hank's balanced salt solution followed by centrifugation at 250g for 4 min. The pellet was resuspended in 2 ml of 35% BSA, and islets were separated using a Histopaque (Sigma) gradient.

**Real-time fluorescence-based RT-PCR.** Total RNA was extracted according to the method of Chomczynski and Sacchi (22). One-step reverse RT-PCR was carried out using the Gold RT-PCR kit from Perkin Elmer Biosystems and an ABI Prism 7700 Sequence detector equipped with a thermocycler (Taqman Technology) and a cooled charged-coupled device camera to detect fluorescence emission over a range of wavelengths (500–650 nm), as previously described by our laboratory (13). Relative expression levels of each gene of interest are expressed as cycle time ( $C_t$ ), i.e., cycle number required for the fluorescence signal of a gene to reach a defined threshold level. The smaller the  $C_t$ , the more abundantly expressed the gene is. The difference between two  $C_t$  values for two genes of interest,  $n$ , denotes a  $2^n$  fold difference in expression levels between the two genes.

**Insulin secretion studies.** Islets were cultured in RPMI media containing 10% FBS and 11.1 mmol/l glucose for 24 h immediately after isolation. The day after isolation, islets were transferred to experimental medium (RPMI media with 0.2% FBS with or without drug) for another 24 h. Static incubation was then performed on the following day. Insulin levels in the Krebs-Ringer buffer samples collected from 1-h static incubations were measured either by radioimmunoassay as previously described (23) or by using a Sensitive Rat Insulin RIA kit (Linco Research, St. Louis, MO).

**Nuclear protein extraction from islets and electromobility shift assays.** Cell lysis was performed in buffer containing 20 mmol/l HEPES, 10 mmol/l KCl, 1 mmol/l EDTA, 10% glycerol, 0.2% IGEPAL (Rhone-Poulenc, Bridgewater, NJ), and protease inhibitors. After lysis and separation from the cytoplasmic fraction by centrifugation, the nuclear pellet was resuspended in buffer containing 420 mmol/l NaCl, 20 mmol/l HEPES, 10 mmol/l KCl, 1 mmol/l EDTA, 20% glycerol, and protease inhibitors. Nuclear proteins were separated out by centrifugation and concentrated using the Microcon 30 column (Millipore, Bedford, MA). Protein concentrations were measured using the Bradford protein assay method. The sense strand of oligonucleotides specific for the NF- $\kappa$ B sequence in the COX-2 promoter (5'-GCGGGAGAGGGGATTCCTGCGGCCCG-3') was end-labeled by T4 polynucleotide kinase (Invitrogen Life Technologies, Carlsbad, CA) using  $\gamma$ - $^{32}$ P[ATP] and annealed to its complementary antisense strand (5'-CGGGGCCGAGGGAATCCCTCTCCC GC). Unincorporated oligonucleotides were removed using a 5'-3' Select D column (5'Prime-3'Prime, Boulder, CO). Radioactive incorporation was determined by counting a 1- $\mu$ l aliquot in a scintillation counter, and 35,000 cpm was used per reaction. Nuclear protein extracts (10  $\mu$ g) were incubated in buffer containing 50 mmol/l Tris, 500 mmol/l NaCl, 5 mmol/l dithiothreitol, 5 mmol/l EDTA, 20% glycerol, and 0.4  $\mu$ g/ $\mu$ l sonicated salmon sperm DNA with radiolabeled probe for 30 min at room temperature. Supershift reactions were done by incubating nuclear proteins with the p65 NF- $\kappa$ B antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 25 min at room temperature before the addition of radiolabeled probe for an additional 30 min at room temperature. After incubation reactions, protein samples were separated on a 4.5% acrylamide gel and then vacuum dried. The level of NF- $\kappa$ B DNA binding activity was measured by exposing the gels to a phosphor screen and scanned using the Cyclone phosphorimager (Packard Instruments, Meriden, CT).

**Expression of data and statistics.** Data are reported as means  $\pm$  SE. Statistical comparisons were performed using Student's  $t$  test and Bonferroni post hoc analysis of variance test with  $P < 0.05$  considered as significant.

## RESULTS

**EP3 gene expression in rat islets, kidney, liver, epididymal fat, and skeletal muscle.** The EP3 receptor type was the most abundantly expressed in islets, followed by EP1 and EP2, then EP4. The difference in the  $C_t$  values for the EP3 ( $C_t = 25$ ) and EP1 ( $C_t = 27$ ) receptor type in the representative graph shown in Fig. 1 indicates a  $2^2$  or four-fold difference in expression levels between the two receptor types. Similar relative expression levels of EP receptor types were found in liver, kidney, and fat but not skeletal muscle, in which EP2 was the most abundantly expressed receptor type and EP3 was barely detectable (Fig. 1).

**EP3 receptor and PGE<sub>2</sub> action in islets.** Of the four major EP receptor types, only EP3 has the postreceptor action of decreasing cAMP (14,15,24). To test the hypothesis that the EP3 receptor mediates PGE<sub>2</sub>-dependent decreases in insulin secretion, we treated islets with misoprostol, an agonist for EP3 (25,26), for 24 h to parallel studies with IL-1 $\beta$ . After a 24-h preincubation with misoprostol, glucose-stimulated insulin secretion during a 1-h static incubation was

compared with that in islets treated for the same amount of time with either vehicle or exogenous PGE<sub>2</sub> (100  $\mu$ mol/l). Glucose (22.2 mmol/l) stimulated insulin secretion almost threefold over basal levels. Misoprostol decreased glucose-stimulated insulin secretion significantly ( $P < 0.001$ ) (Fig. 2A). To determine whether the inhibitory effects of misoprostol on insulin secretion were mediated through cAMP, we pretreated islets with pertussis toxin (10 ng/ml). Pertussis toxin irreversibly ADP-ribosylates the G<sub>i/o</sub> protein, preventing G<sub>i/o</sub>-dependent inhibitory effects on adenylate cyclase, thereby blocking G<sub>i/o</sub> inhibition of adenylate cyclase. Pretreatment with pertussis toxin prevented the misoprostol-dependent inhibition of glucose-stimulated insulin secretion (Fig. 2B). Treatment with a second EP3 agonist, sulprostone, showed the same result as with misoprostol. Because no specific antagonist of the EP3 receptor is available for further confirmation that the EP3 receptor mediates PGE<sub>2</sub>-dependent inhibition of insulin secretion, we asked whether the EP1 receptor type, the second most abundant receptor type in the islet, also contributed to the effects of PGE<sub>2</sub>. Concurrent treatment of islets with IL-1 $\beta$  and AH-6809, an EP1-specific antagonist, did not prevent the IL-1 $\beta$ -dependent decreases in glucose-induced insulin secretion (Fig. 3). As a positive control for the responsiveness of islets to inhibitors of IL-1 $\beta$  effects, we also treated islets with two COX-2-specific inhibitors, NS-398 or SC-236, for 24 h concurrently with IL-1 $\beta$ . As with previous studies (13), both NS-398 and SC-236 prevented the IL-1 $\beta$ -dependent decrease in glucose-stimulated insulin secretion. Morphological assessment of islets by light microscopy did not reveal any signs of cellular toxicity as a result of treatment with either misoprostol or AH-6809 for 24 h. Treatment with AH-6809 alone for 24 h did not affect glucose-stimulated insulin secretion.

**Sites of action of sodium salicylate as an inhibitor of IL-1 $\beta$ -dependent decreases in glucose-stimulated insulin secretion.** To ascertain whether pretreatment with sodium salicylate would prevent IL-1 $\beta$ 's negative effects on  $\beta$ -cell function, we pretreated islets with sodium salicylate (0.002, 0.2, and 20 mg/dl) for 45 min before the addition of IL-1 $\beta$  (5 ng/ml). These concentrations of sodium salicylate were chosen because they approximate plasma levels that partially restore defective glucose-induced insulin secretion in humans with diabetes (2). Islets were then cultured for an additional 24 h with both sodium salicylate and IL-1 $\beta$  before static incubations were performed to assess glucose-stimulated insulin secretion. Glucose (22.2 mmol/l) stimulated insulin secretion threefold over basal levels. Exposure of islets to IL-1 $\beta$  (5 ng/ml) completely inhibited glucose-stimulated insulin secretion to basal levels. Pretreatment with sodium salicylate (20 mg/dl) before the addition of IL-1 $\beta$  prevented the IL-1 $\beta$ -dependent decrease in insulin secretion such that glucose-stimulated insulin secretion was not significantly different from control (Fig. 4). No effect on either glucose-stimulated or basal insulin secretion was observed after treatment with sodium salicylate (up to 20 mg/dl) for 24 h.

The effects of sodium salicylate on IL-1 $\beta$  activation of NF- $\kappa$ B binding activity in nuclear protein extracts was determined using electromobility shift assays. Exposure of islets to IL-1 $\beta$  increased NF- $\kappa$ B binding activity over control levels, with maximum activity observed after 1 h of IL-1 $\beta$  treatment (Fig. 5). Pretreatment with sodium salicylate

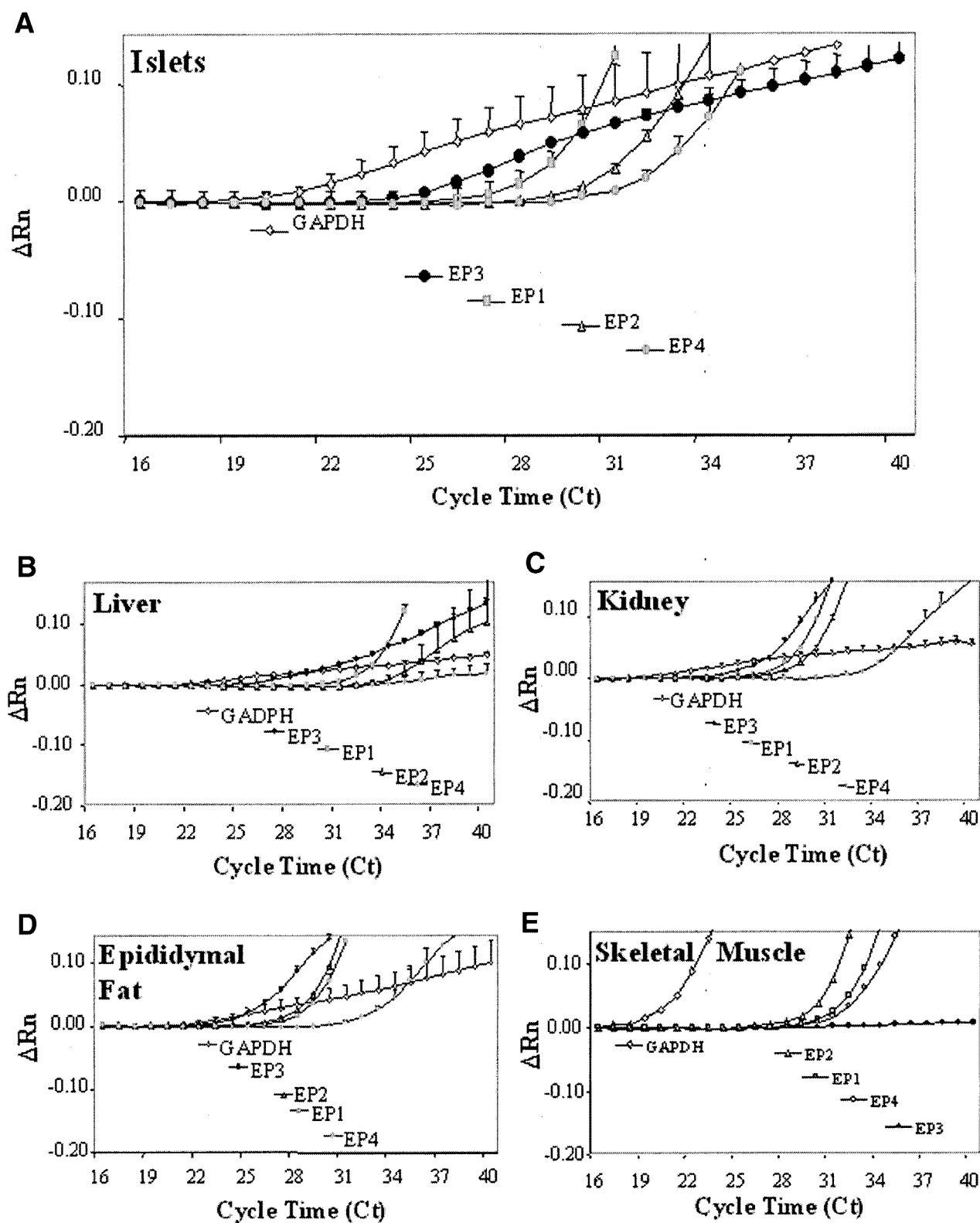
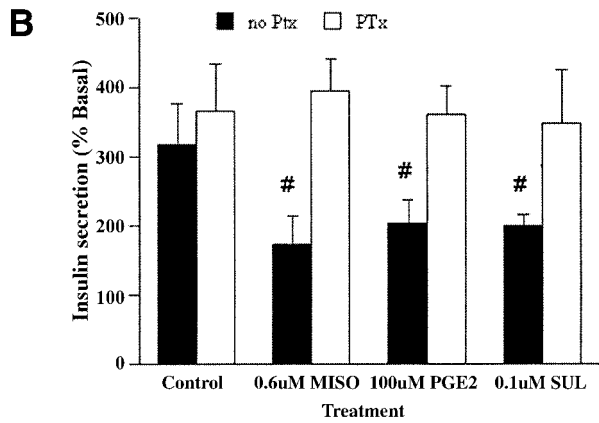
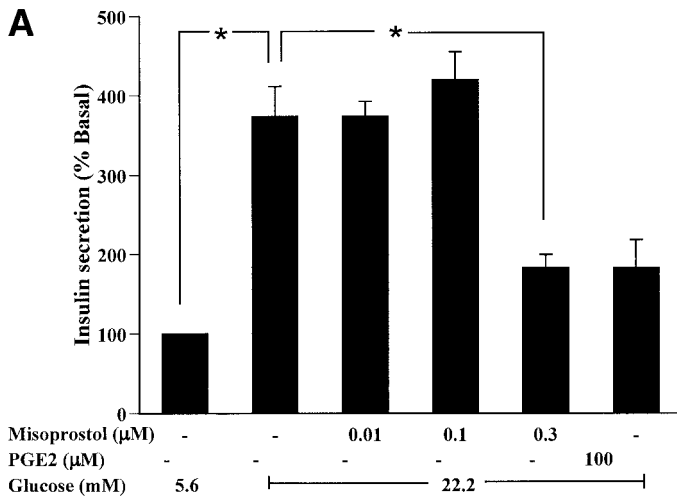


FIG. 1. Abundance of EP receptor types in rat islets (A), liver (B), kidney (C), epididymal fat (D), and skeletal muscle (E). Relative expression levels of the four major EP receptor types were identified in rat tissues using fluorescence-based RT-PCR with Taqman probes and primers. EP3 is most dominantly expressed in islets, followed by EP1, EP2, and EP4, because its fluorescence signal reached threshold level with the smallest  $C_t$  value. EP3 is also dominantly expressed in liver, kidney, and epididymal fat, whereas EP2 is the dominant receptor type in skeletal muscle. Profiles are representative of experiments with tissue isolated from at least  $n = 3$  animals.

plate before the addition of IL-1 $\beta$  prevented the increase in NF- $\kappa$ B binding activity, whereas treatment with sodium salicylate alone had no effect on NF- $\kappa$ B binding activity.

Binding was supershifted by antibodies directed against the p65 form of NF- $\kappa$ B. We next determined whether sodium salicylate also prevents IL-1 $\beta$  stimulation of COX-2

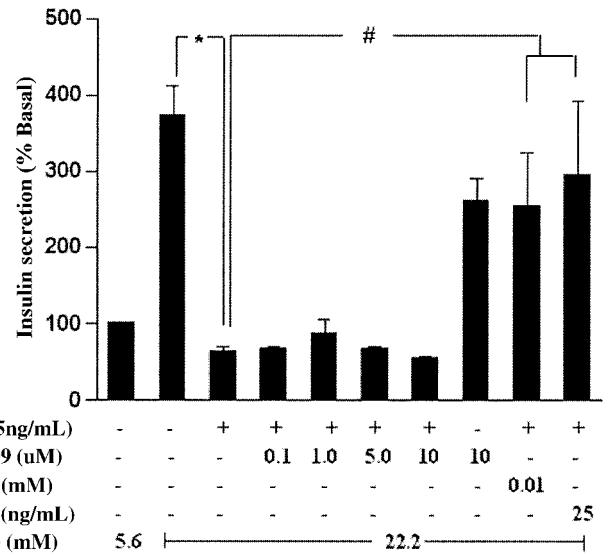


**FIG. 2.** Misoprostol mimics the negative effects of exogenous PGE<sub>2</sub> on insulin secretion through cAMP-dependent pathways. **A:** Insulin secretion from Wistar rat islets was stimulated 3.5-fold over basal levels by glucose (22.2 mmol/l). Treatment with misoprostol (0.3 μmol/l), an EP3 agonist, for 24 h inhibited glucose-stimulated insulin secretion to the same extent as the addition of exogenous PGE<sub>2</sub> (100 μmol/l) for 24 h (*n* = 6). \**P* < 0.001. **B:** Pretreatment with pertussis toxin (10 ng/ml) for 24 h before and concurrently with the 24-h misoprostol treatment blocked the inhibitory effects of misoprostol as well as PGE<sub>2</sub> on glucose-stimulated insulin secretion (*n* = 4). #*P* < 0.05 PTx-treated (□) compared with No PTx-treated controls (■). MISO, misoprostol; SUL, sulprostone.

and EP3 receptor gene expression, via blocking IL-1β activation of NF-κB. Wistar rat islets were pretreated with sodium salicylate (20 mg/dl) for 45 min and then exposed to IL-1β (5 ng/ml) for an additional 2 h. RNA was extracted and analyzed by Taqman-based RT-PCR. IL-1β alone increased COX-2 and EP3 receptor gene expression 3.5- and 2-fold over control levels, respectively. Pretreatment with sodium salicylate before the addition of IL-1β significantly decreased the induction by IL-1β of both COX-2 and EP3 receptor gene expression (Fig. 6A and B, respectively).

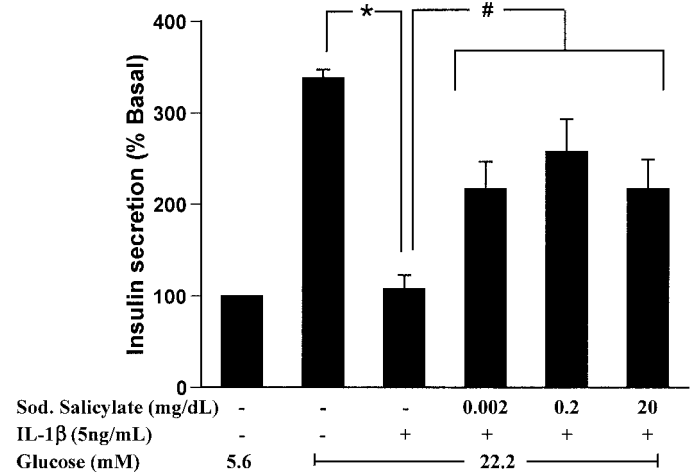
**DISCUSSION**

We performed these experiments to identify prostaglandin-related mechanisms whereby sodium salicylate might prevent IL-1β's adverse effects on pancreatic β-cell function. Our results indicate that EP3 is the most abundant EP receptor type in the islet, liver, kidney, and epididymal fat and that it is the least abundant in skeletal muscle, in

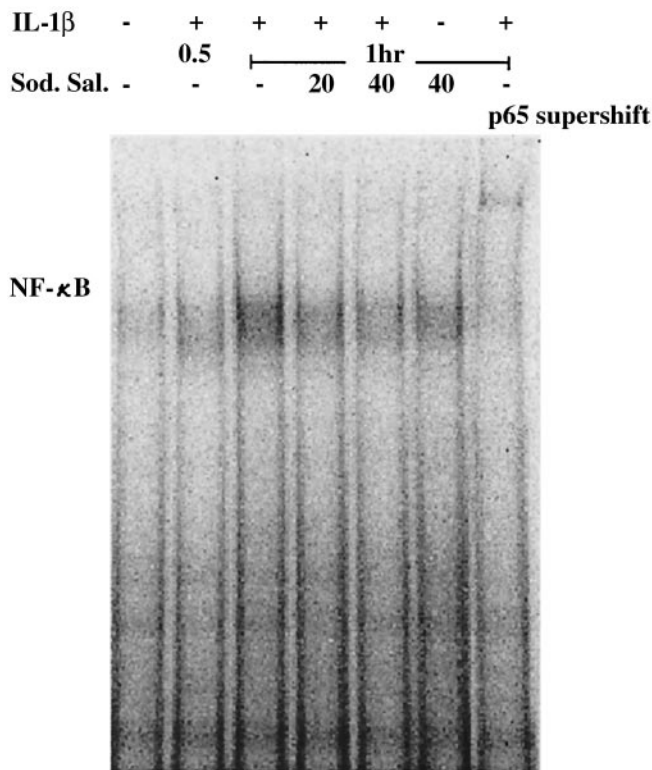


**FIG. 3.** AH-6809, an EP1 antagonist, does not block the inhibitory effects of IL-1β on glucose-induced insulin secretion. Insulin secretion from Wistar rat islets was stimulated 3.5-fold over basal levels by glucose (22.2 mmol/l). Twenty-four-hour concurrent treatment with IL-1β and increasing concentrations with AH-6809 did not significantly affect IL-1β inhibition of glucose-induced insulin secretion. Concurrent treatment with NS-398 or SC-236, to confirm that the IL-1β inhibition of glucose-stimulated insulin secretion was mediated by PGE<sub>2</sub>, completely inhibited IL-1β-dependent decreases in glucose-induced insulin secretion. \**P* < 0.001; #*P* < 0.05; *n* = 3.

which EP2 is the most abundant receptor type. The findings with nonislet tissues are in general agreement with past findings using conventional Northern blot analysis (15,26). The EP3 agonist misoprostol at a concentration previously shown to inhibit forskolin-induced cAMP increases in EP3-expressing CHO cells (25,26) inhibited glucose-stimulated insulin secretion. Similar results were obtained using a second EP3 agonist, sulprostone. The effects of misoprostol on insulin secretion were mediated by G<sub>i</sub>-dependent decreases in cAMP because pretreatment



**FIG. 4.** Sodium salicylate blocks IL-1β-dependent decreases in glucose-stimulated insulin secretion. Glucose (22.2 mmol/l) stimulated insulin secretion in Wistar rat islets with or without previous treatment with sodium salicylate (20 mg/dl). Treatment with IL-1β alone for 24 h reduced insulin secretion to basal levels (\**P* < 0.001). Pretreatment with sodium salicylate for 45 min before the addition of IL-1β partially prevented this reduction in insulin secretion (#*P* < 0.05). There were no differences found in the efficacy of sodium salicylate over the 0.002–20 mg/dl concentration range.



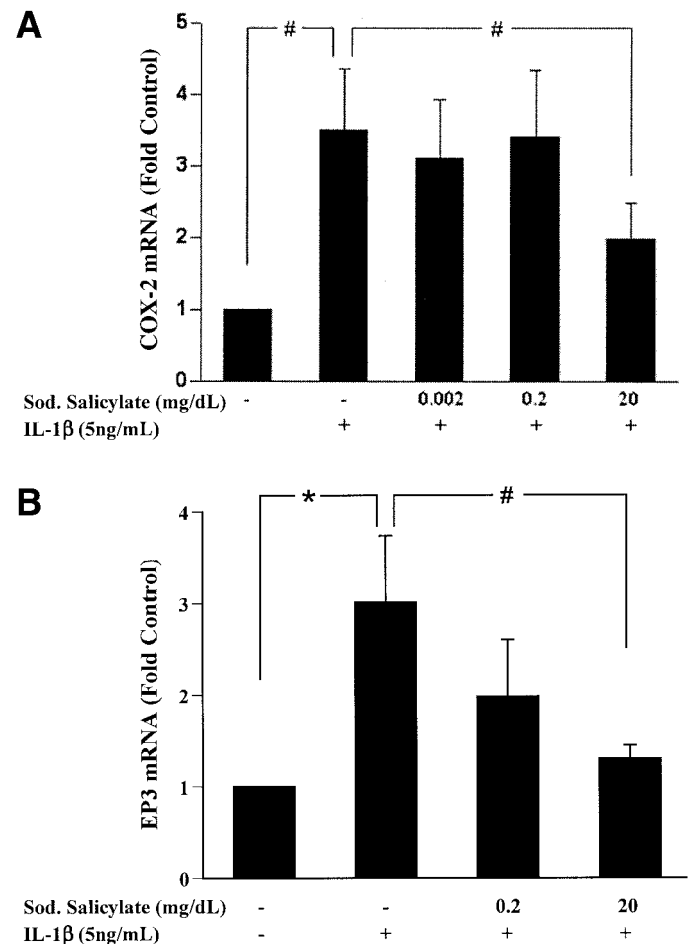
**FIG. 5.** Sodium salicylate inhibits IL-1 $\beta$  activation of NF- $\kappa$ B. Wistar rat islets in RPMI containing glucose (22.2 mmol/l, control) were exposed to IL-1 $\beta$  (5 ng/ml) for 1 h with or without a 45-min pretreatment with sodium salicylate (20 and 40 mg/dl). The ability of sodium salicylate to block NF- $\kappa$ B binding was evaluated using electromobility shift assay. Pretreatment with sodium salicylate (for 45 min before addition of IL-1 $\beta$ ) decreased the level of NF- $\kappa$ B binding induced by IL-1 $\beta$ . Lane 1, 0 h control; lane 2, 0.5 h IL-1 $\beta$  treated; lane 3, 1 h IL-1 $\beta$  treated; Lane 4, 1 h IL-1 $\beta$ , 20 mg/dl sodium salicylate treated; lane 5, 1 h IL-1 $\beta$ , 40 mg/dl sodium salicylate treated; lane 6, 40 mg/dl sodium salicylate control; lane 7, supershift with p65 NF- $\kappa$ B. Representative radiograph from  $n = 3$  experiments.

with pertussis toxin, which ADP-ribosylates and inhibits the activation of G $_i$ , blocked the effect of misoprostol. Pretreatment with pertussis toxin has been shown to inhibit the adverse effects of PGE $_2$  on glucose-stimulated insulin secretion through ADP ribosylation of the G $_i$  components of adenylate cyclase (27). No EP3 antagonist is available for further confirmation of the EP3 receptor's involvement. The second most abundant receptor type in the islet, EP1, is unlikely to be involved in IL-1 $\beta$ /PGE $_2$  inhibitory effects on insulin secretion because treatment with the EP1 antagonist AH-6809 did not prevent decreases in glucose-stimulated insulin secretion. Sodium salicylate inhibited IL-1 $\beta$ -induced NF- $\kappa$ B and also decreased EP3 and COX-2 gene expression in islets. These findings support the functional dependence of IL-1 $\beta$  as an inhibitor of  $\beta$ -cell function on the endogenous synthesis and action of PGE $_2$  and establish a new site of action in the islet for the beneficial effects of sodium salicylate on insulin secretion.

Insulin is synthesized in and secreted from pancreatic islet  $\beta$ -cells both basally and in response to a number of secretagogues. The main targets for insulin action are fat, liver, and skeletal muscle, whereas the major sites of insulin degradation are liver and kidney. The findings of EP3 receptor dominance in fat, liver, and kidney and EP2 receptor dominance in skeletal muscle are consistent with

known actions of PGE $_2$  in these tissues. In fat, PGE $_2$  decreases adenylate cyclase and cAMP generation and inhibits hormone-induced lipolysis (28–32). In liver, PGE $_2$  also inhibits adenylate cyclase activity and decreases glucagon-stimulated glycogenolysis (33,34). In kidney, PGE $_2$  has been shown to decrease cAMP levels and vasopressin-induced water resorption by the collecting tubules (35–37). In skeletal muscle, the greater abundance of the EP1 and EP2 receptor types is consistent with the reported effects of PGE $_2$  to influence both contraction and relaxation of muscle fibers (14).

Potential interrelationships between IL-1 $\beta$  and PGE $_2$  as inhibitors of insulin secretion have been examined for more than a decade. IL-1 $\beta$  and PGE $_2$  are known inhibitors of  $\beta$ -cell function, especially under conditions involving stimulatory glucose concentrations (4,8,9,13,38–41). COX is found in most tissues in both constitutive and inducible forms, termed COX-1 and -2, respectively. The pancreatic islet is relatively unique among other tissues because it expresses COX-2 as the dominant form even under basal conditions (42). We showed previously that inhibition of

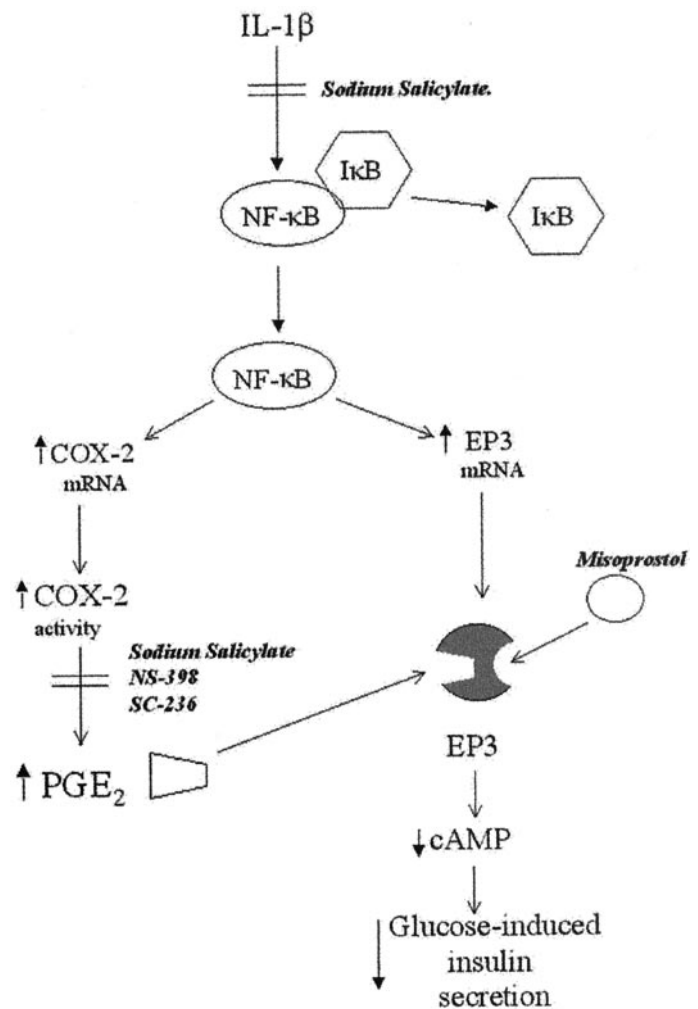


**FIG. 6.** IL-1 $\beta$ -dependent induction of COX-2 and EP3 gene expression is inhibited by sodium salicylate. Wistar rat islets incubated in RPMI containing glucose (22.2 mmol/l, control) were exposed to IL-1 $\beta$  (5 ng/ml) for 2 h with or without a 45-min pretreatment with sodium salicylate (20 mg/dl). RNA was evaluated using fluorescence-based RT-PCR. IL-1 $\beta$  increased COX-2 mRNA levels at least threefold (A) and increased EP3 mRNA levels twofold over control levels (B). Pretreatment with sodium salicylate prevented the IL-1 $\beta$ -dependent induction of both COX-2 ( $n = 5$ ; A) and EP3 mRNA ( $n = 3$ ; B). # $P < 0.05$ ; \* $P < 0.001$ .

COX-2 activity by two specific antagonists prevents IL-1 $\beta$  from inhibiting insulin secretion (13). Of all the prostaglandins, leukotrienes, and hydroxyecosatetraenoic acid products formed from arachidonic acid by COX, PGE<sub>2</sub> is the product shown most reproducibly to modulate  $\beta$ -cell function (43). PGE<sub>2</sub> was shown previously to bind to a single class of PGE-specific receptors in the islet whose postreceptor activity activates G<sub>i/o</sub> proteins and decreases adenylate cyclase activity and cAMP levels (27). Of the four known receptors, only EP3 has a postreceptor effect that is consistent with decreases in insulin secretion, i.e., inhibition of adenylate cyclase activation. In the work described herein, we confirm our previous observation that EP3 is the most abundant EP receptor type expressed in the islet but also that misoprostol, a drug that binds to the EP3 receptor, mimics the inhibitory actions of PGE<sub>2</sub> on glucose-induced secretion.

Sodium salicylate has been known for >30 years to be an inhibitor of COX activity (44,45) and has been demonstrated to improve defective insulin secretion in patients with diabetes (2,46,47). This drug has been reported to inhibit dissociation of NF- $\kappa$ B from NF- $\kappa$ B/I $\kappa$ B complexes (17,18), thereby preventing transport of NF- $\kappa$ B from the cytoplasm to the nucleus of cells, where it stimulates COX-2 and EP receptor transcription (19,20). The current studies present novel results indicating that sodium salicylate inhibits NF- $\kappa$ B activation in islets and also inhibits IL-1 $\beta$ -induced gene expression of islet COX-2 and EP3 receptor mRNA. In addition, the sodium salicylate effect of blocking IL-1 $\beta$  inhibition of glucose-induced insulin secretion seems to be incomplete. This partial effect of sodium salicylate may relate to the fact that sodium salicylate only partially inhibited IL-1 $\beta$  induction of COX-2 mRNA. Inhibition of mRNA induction would not affect posttranscriptional products or the enzymatic activity of COX-2 proteins, which exist at much higher levels in the islets than the COX-1 isoform (42). Only specific inhibitors of COX-2 activity would be able to circumvent completely any increases in PGE<sub>2</sub> production that would lead to inhibition of insulin secretion, as we have previously reported (13). Other nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin, have also been found to be useful in preventing the adverse effects of IL-1 $\beta$  on islet function by interfering with the production of nitric oxide (48,49). Aspirin, unlike sodium salicylate, does not interfere with the IL-1 $\beta$ -dependent NF- $\kappa$ B activation and increases in inducible nitric oxide synthase mRNA levels (48,50). These differences in the mechanisms of action of the different NSAIDs may be due in part to their acetylation states. In addition, although sodium salicylate at higher supraphysiological concentrations has been shown to affect basal insulin secretion (48,51), no significant effects were observed up to 20 mg/dl in these studies.

The current findings, coupled with others that we reported previously (42), suggest the hypothetical schema shown in Fig. 7 for the prostaglandin-dependent mechanism through which sodium salicylate interferes with IL-1 $\beta$ 's adverse effects on pancreatic  $\beta$ -cell function. IL-1 $\beta$  is shown to increase gene expression of COX-2 and EP3 in pancreatic islets by virtue of its ability to activate NF- $\kappa$ B. These events lead to increased synthesis of PGE<sub>2</sub> and EP3 receptor protein. Binding of PGE<sub>2</sub> to EP3 has been shown



**FIG. 7. Hypothesis: prostaglandin-dependent mechanism through which sodium salicylate interferes with IL-1 $\beta$ 's adverse effects on pancreatic  $\beta$ -cell function includes IL-1 $\beta$  activation of COX-2 and EP3 receptor gene expression via NF- $\kappa$ B activation. Increases in COX-2 expression and activity results in an increase in intra-islet PGE<sub>2</sub> levels. PGE<sub>2</sub> binds to the EP3 receptor type, whose postreceptor activity results in a decrease in glucose-stimulated insulin secretion. Sodium salicylate prevents IL-1 $\beta$  induction of COX-2 and EP3 mRNAs by inhibiting NF- $\kappa$ B activation and also inhibits COX-2 activity.**

by us to decrease islet cAMP levels (27), thereby decreasing glucose-induced insulin secretion. Interference with these events by sodium salicylate occurs at the site of IL-1 $\beta$  activation of NF- $\kappa$ B and of COX-2 enzymatic activity. We propose that these actions of sodium salicylate have relevance to its known ability to improve insulin secretion in patients with diabetes.

#### ACKNOWLEDGMENTS

This study was funded by an ADA Mentorship Award to R.P.R.

We thank Alma Rivera, Scott Holland, Elizabeth Oseid, and Jamie Harmon for technical assistance and Dr. Vincent Poitout for careful reading of the manuscript and scientific discussions.

#### REFERENCES

1. Burr IM, Sharp R: Effects of prostaglandin E1 and of epinephrine on the dynamics of insulin release in vitro. *Endocrinology* 94:835-839, 1974
2. Robertson RP, Chen M: A role for prostaglandin E in defective insulin

- secretion and carbohydrate intolerance in diabetes mellitus. *J Clin Invest* 60:747-753, 1977
3. Chen M, Robertson RP: Effects of prostaglandin synthesis inhibitors on human insulin secretion and carbohydrates tolerance. *Prostaglandins* 18:557-567, 1979
  4. Metz SA, Robertson RP, Fujimoto WY: Inhibition of prostaglandin E synthesis augments glucose-induced insulin secretion in cultured pancreas. *Diabetes* 30:551-557, 1981
  5. Giugliano D, Di Pinto P, Torella R, Frascolla N, Saccomanno F, Passariello N, D'Onofrio F: A role for endogenous prostaglandin E in biphasic pattern of insulin release in humans. *Am J Physiol* 245:E591-E597, 1983
  6. Comens PG, Wolf BA, Unanue ER, Lacy PE, McDaniel ML: Interleukin 1 is potent modulator of insulin secretion from isolated rat islets of Langerhans. *Diabetes* 36:963-970, 1987
  7. Hughes JH, Easom RA, Wolf BA, Turk J, McDaniel ML: Interleukin 1-induced prostaglandin E2: accumulation by isolated pancreatic islets. *Diabetes* 38:1251-1257, 1989
  8. Palmer JP, Helqvist S, Spinass GA, Molvig J, Mandrup-Poulsen T, Andersen HU, Nerup J: Interaction of beta-cell activity and IL-1 concentration and exposure time in isolated rat islets of Langerhans. *Diabetes* 38:1211-1216, 1989
  9. Zawulich WS, Zawulich KC, Rasmussen H: Interleukin-1 alpha exerts glucose-dependent stimulatory and inhibitory effects on islet cell phosphoinositide hydrolysis and insulin secretion. *Endocrinology* 124:2350-2357, 1989
  10. Eizirik DL, Bendtzen K, Sandler S: Short exposure of rat pancreatic islets to interleukin-1 beta induces a sustained but reversible impairment in beta-cell function: influence of protease activation, gene transcription, and protein synthesis. *Endocrinology* 128:1611-1616, 1991
  11. Sjöholm A: Prostaglandins inhibit pancreatic beta-cell replication and long-term insulin secretion by pertussis toxin-insensitive mechanisms but do not mediate the actions of interleukin-1 beta. *Biochim Biophys Acta* 1313:106-110, 1996
  12. Dayer-Metroz MD, Wollheim CB, Seckinger P, Dayer JM: A natural interleukin 1 (IL-1) inhibitor counteracts the inhibitory effect of IL-1 on insulin production in cultured rat pancreatic islets. *J Autoimmun* 2:163-171, 1989
  13. Tran PO, Gleason CE, Poitout V, Robertson RP: Prostaglandin E(2) mediates inhibition of insulin secretion by interleukin-1 $\beta$ . *J Biol Chem* 274:31245-31248, 1999
  14. Coleman RA, Smith WL, Narumiya S: International Union of Pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. *Pharmacol Rev* 46:205-229, 1994
  15. Negishi M, Sugimoto Y, Ichikawa A: Prostaglandin E receptors. *J Lipid Mediat Cell Signal*. 12:379-391, 1995
  16. Mitchell JA, Akaraseenont P, Thiernemann C, Flower RJ, Vane JR: Selectivity of nonsteroidal antiinflammatory drugs as inhibitors of constitutive and inducible cyclooxygenase. *Proc Natl Acad Sci U S A* 90:11693-11697, 1993
  17. Alpert D, Vilcek J: Inhibition of I $\kappa$ B kinase activity by sodium salicylate in vitro does not reflect its inhibitory mechanism in intact cells. *J Biol Chem* 275:10925-10929, 2000
  18. Kopp E, Ghosh S: Inhibition of NF- $\kappa$ B by sodium salicylate and aspirin. *Science* 265:956-959, 1994
  19. Xie W, Merrill JR, Bradshaw WS, Simmons DL: Structural determination and promoter analysis of the chicken mitogen-inducible prostaglandin G/H synthase gene and genetic mapping of the murine homolog. *Arch Biochem Biophys* 300:247-252, 1993
  20. Arakawa T, Laneuville O, Miller CA, Lakkides KM, Wingerd BA, DeWitt DL, Smith WL: Prostanoid receptors of murine NIH 3T3 and RAW 264.7 cells: structure and expression of the murine prostaglandin EP4 receptor gene. *J Biol Chem* 271:29569-29575, 1996
  21. Xu XM, Sansores-Garcia L, Chen XM, Matijevic-Aleksic N, Du M, Wu KK: Suppression of inducible cyclooxygenase 2 gene transcription by aspirin and sodium salicylate. *Proc Natl Acad Sci U S A* 96:5292-5297, 1999
  22. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156-159, 1987
  23. Zhang HJ, Walseth TF, Robertson RP: Insulin secretion and cAMP metabolism in HIT cells: reciprocal and serial passage-dependent relationships. *Diabetes* 38:44-48, 1989
  24. Robertson R: Molecular regulation of prostaglandin synthesis: implications for endocrine systems. *Trends Endocrinol Metab* 6:293-297, 1995
  25. Negishi M, Harazono A, Sugimoto Y, Hazato A, Kurozumi S, Ichikawa A: TEI-3356, a highly selective agonist for the prostaglandin EP3 receptor. *Prostaglandins* 48:275-283, 1994
  26. Boie Y, Stocco R, Sawyer N, Slipetz DM, Ungrin MD, Neuschaefer-Rube F, Puschel GP, Metters KM, Abramovitz M: Molecular cloning and characterization of the four rat prostaglandin E2 prostanoid receptor subtypes. *Eur J Pharmacol* 340:227-241, 1997
  27. Robertson RP, Tsai P, Little SA, Zhang HJ, Walseth TF: Receptor-mediated adenylate cyclase-coupled mechanism for PGE2 inhibition of insulin secretion in HIT cells. *Diabetes* 36:1047-1053, 1987
  28. Butcher RW, Baird CE: Effects of prostaglandins on adenosine 3',5'-monophosphate levels in fat and other tissues. *J Biol Chem* 243:1713-1717, 1968
  29. Steinberg D: Hormonal control of lipolysis in adipose tissue. *Adv Exp Med Biol* 26:77-88, 1972
  30. Wieser PB, Fain JN: Insulin, prostaglandin E1, phenylisopropyladenosine and nicotinic acid as regulators of fat cell metabolism. *Endocrinology* 96:1221-1225, 1975
  31. Richelsen B, Pedersen SB: Antilipolytic effect of prostaglandin E2 in perfused rat adipocytes. *Endocrinology* 121:1221-1226, 1987
  32. Strong P, Coleman RA, Humphrey PP: Prostanoid-induced inhibition of lipolysis in rat isolated adipocytes: probable involvement of EP3 receptors. *Prostaglandins* 43:559-566, 1992
  33. Brass EP, Garrity MJ, Robertson RP: Inhibition of glucagon-stimulated hepatic glycogenolysis by E-series prostaglandins. *FEBS Lett* 169:293-296, 1984
  34. Melien O, Winsnes R, Refsnes M, Gladhaug IP, Christoffersen T: Pertussis toxin abolishes the inhibitory effects of prostaglandins E1, E2, I2 and F2 alpha on hormone-induced cAMP accumulation in cultured hepatocytes. *Eur J Biochem* 172:293-297, 1988
  35. Zusman RM, Keiser HR, Handler JS: Vasopressin-stimulated prostaglandin E biosynthesis in the toad urinary bladder: effect of water flow. *J Clin Invest* 60:1339-1347, 1977
  36. Garcia-Perez A, Smith WL: Apical-basolateral membrane asymmetry in canine cortical collecting tubule cells: bradykinin, arginine vasopressin, prostaglandin E2 interrelationships. *J Clin Invest* 74:63-74, 1984
  37. Sonnenburg WK, Smith WL: Regulation of cyclic AMP metabolism in rabbit cortical collecting tubule cells by prostaglandins. *J Biol Chem* 263:6155-6160, 1988
  38. Robertson RP: Prostaglandins as modulators of pancreatic islet function. *Diabetes* 28:942-948, 1979
  39. Robertson RP: Eicosanoids as pluripotential modulators of pancreatic islet function. *Diabetes* 37:367-370, 1988
  40. Spinass GA, Palmer JP, Mandrup-Poulsen T, Andersen H, Nielsen JH, Nerup J: The bimodal effect of interleukin 1 on rat pancreatic beta-cells—stimulation followed by inhibition—depends upon dose, duration of exposure, and ambient glucose concentration. *Acta Endocrinol (Copenh)* 119:307-311, 1988
  41. Eizirik DL, Welsh M, Strandell E, Welsh N, Sandler S: Interleukin-1 beta depletes insulin messenger ribonucleic acid and increases the heat shock protein hsp70 in mouse pancreatic islets without impairing the glucose metabolism. *Endocrinology* 127:2290-2297, 1990
  42. Sorli CH, Zhang HJ, Armstrong MB, Rajotte RV, Maclouf J, Robertson RP: Basal expression of cyclooxygenase-2 and nuclear factor-interleukin 6 are dominant and coordinately regulated by interleukin 1 in the pancreatic islet. *Proc Natl Acad Sci U S A* 95:1788-1793, 1998
  43. Robertson RP: Arachidonic acid metabolite regulation of insulin secretion. *Diabetes Metab Rev* 2:261-296, 1986
  44. Vane JR: Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nat New Biol* 231:232-235, 1971
  45. Smith JB, Willis AL: Aspirin selectively inhibits prostaglandin production in human platelets. *Nat New Biol* 231:235-237, 1971
  46. Robertson RP, Chen M: Modulation of insulin secretion in normal and diabetic humans by prostaglandin E and sodium salicylate. *Trans Assoc Am Physicians* 90:353-365, 1977
  47. Chen M, Robertson RP: Restoration of the acute insulin response by sodium salicylate: a glucose dose-related phenomenon. *Diabetes* 27:750-756, 1978
  48. Kwon G, Hill JR, Corbett JA, McDaniel ML: Effects of aspirin on nitric oxide formation and de novo protein synthesis by RINm5F cells and rat islets. *Mol Pharmacol* 52:398-405, 1997
  49. Kwon G, Corbett JA, Hauser S, Hill JR, Turk J, McDaniel ML: Evidence for involvement of the proteasome complex (26S) and NF $\kappa$ B in IL-1 $\beta$ -induced nitric oxide and prostaglandin production by rat islets and RINm5F cells. *Diabetes* 47:583-591, 1998
  50. Amin AR, Vyas P, Attur M, Leszczynska-Piziak J, Patel IR, Weissmann G, Abramson SB: The mode of action of aspirin-like drugs: effect on inducible nitric oxide synthase. *Proc Natl Acad Sci U S A* 92:7926-7930, 1995
  51. Metz S, Fujimoto W, Robertson RP: Modulation of insulin secretion by cyclic AMP and prostaglandin E: the effects of theophylline, sodium salicylate and tolbutamide. *Metabolism* 31:1014-1022, 1982