Lipoxygenase Pathway in Islet Endocrine Cells

OXIDATIVE METABOLISM OF ARACHIDONIC ACID PROMOTES INSULIN RELEASE

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ABSTRACT Metabolism of arachidonic acid (AA) via the cyclooxygenase pathway reduces glucose-stimulated insulin release. However, metabolism of AA by the lipoxygenase pathway and the consequent effects on insulin secretion have not been simultaneously assessed in the endocrine islet. Both dispersed endocrine cell-enriched pancreatic cells of the neonatal rat, as well as intact islets of the adult rat, metabolized [^{3}H]AA not only to cyclooxygenase products (prostaglandins E_2, $F_{2\alpha}$, and prostacyclin) but also to the lipoxygenase product 12-hydroxyeicosatetraenoic acid (12-HETE). 12-HETE was identified by coelution with authentic tritiated or unlabeled 12-HETE using four high performance liquid chromatographic systems under eight mobile-phase conditions and its identity was confirmed by gas chromatography/mass spectrometry using selected ion monitoring. The predominant effect of exogenous AA (5 μg/ml) was to stimulate insulin release from pancreatic cells grown in monolayer. This effect was concentration- and time-dependent, and reversible. The effect of AA upon insulin release was potentiated by a cyclooxygenase inhibitor (indomethacin) and was prevented by either of two lipoxygenase inhibitors (5,8,11,14-eicosatetraynoic acid [ETYA] and BW755c). In addition, glucose, as well as two structurally dissimilar agents (the calcium ionophore A23187 and bradykinin), which activate phospholipase(s) and thereby release endogenous AA in several cell systems, also stimulated insulin secretion. The effects of glucose, glucagon, bradykinin and high concentrations of A23187 (5 μg/ml) to augment insulin release were blocked or considerably reduced by lipoxygenase inhibitors. However, a lower concentration of the ionophore (0.25 μg/ml), which did not appear to activate phospholipase, was resistant to blockade. Exogenous 12-HETE (up to 2,000 ng/ml) did not alter glucose-induced insulin release. However, the labile intermediate 12-hydroperoxy-ETE increased insulin release. Furthermore, diethylmaleate (which binds intracellular glutathione and thereby impedes conversion of the lipoxygenase intermediates hydroperoxy-ETE and leukotriene A_4 to HETE and leukotriene C_4, respectively) potentiated the effect of glucose and of exogenous AA. Finally, 5,6-epoxy, 8,11,14-eicosatrienoic acid (a relatively stable epoxide analogue of leukotriene A_4) as well as two other epoxy-analogues, potentiated glucose-induced insulin release. We conclude that dual pathways of AA metabolism exist in islet endocrine cells and have opposing regulatory effects on the beta cell—an inhibitory cyclooxygenase cascade and a stimulatory lipoxygenase cascade. Labile products of the latter pathway may play a pivotal role in stimulus-secretion coupling in the islet.

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INTRODUCTION

Arachidonic acid (AA)[1] is enzymatically oxidized in many cells along two major pathways. The first, the

[1] *Abbreviations used in this paper:* AA, arachidonic acid; ACN, acetonitrile; ETYA, 5,8,11,14-eicosatetraynoic acid; GC, gas chromatography; 12-HETE, 12-hydroxyeicosate-

cyclooxygenase (prostaglandin synthetase) cascade, leads to production of the classic prostaglandins (PG) as well as thromboxane and prostacyclin. The second, the lipoxygenase pathway, oxidizes AA to one or more hydroperoxyeicosatetraenoic acids (hydroperoxy-ETE). These hydroperoxy-ETE can be converted either to their respective hydroxy fatty acids (HETE) or to leukotrienes, a family of lipids derived from the unstable epoxide leukotriene A_4 (LTA_4) via hydration or conjugation to glutathione (1). Although several metabolites of the lipoxygenase pathway have marked biological activities in certain systems such as leukocytes (1), the potential role of these metabolites in endocrine systems, and particularly within the islet of Langerhans, has received virtually no attention. We have previously demonstrated that monolayer cultures of neonatal rat pancreatic cells synthesize immunoassayable PGE and that inhibition of PG synthesis by sodium salicylate or ibuprofen augments glucose-induced insulin release (2, 3). Although we have presented data that suggest that inhibition of PG synthesis is sufficient to explain the latter effect (2), it is possible that inhibition of cyclooxygenase could also shunt AA into the lipoxygenase pathway. Metabolites of the latter pathway might then mediate alterations of insulin release. To test this possibility, we have examined whether the lipoxygenase pathway is present in islet endocrine cells, and, if so, whether this pathway modulates insulin release.

METHODS

Chemicals. BW755c was a gift of Dr. P. J. McHale of the Wellcome Research Laboratories (Kent, England) and 5,8,11,14-eicosatetraynoic acid (ETYA) was a gift of Dr. W. B. Scott, of Hoffmann-LaRoche (Nutley, NJ). Nordihydroguaiaretic acid (NDGA), bradykinin triacetate, AA, oleic acid, stearic acid, arachidic acid, diethyl maleate, and delipidated bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO). Indomethacin was obtained from the Upjohn Co. (Kalamazoo, MI). Calcium ionophore (A23187) was purchased from Calbiochem-Behring Corp. (La Jolla, CA). Solvents for high performance liquid chromatography (HPLC) were obtained from Burdick and Jackson (Muskegon, MI). Methylformate (Spectro grade) was obtained from Eastman Kodak Co. (Rochester, NY). 5,6,8,-9,11,12,14,15[^{3}H]12-HETE and 12-hydroperoxy-ETE were generously provided by Dr. W. C. Hubbard (Vanderbilt University, Nashville, TN), and unlabeled authentic 12-HETE was supplied by the Upjohn Co. ^{14}C-labeled trihydroxyeicosatrienoic acids and epoxy-hydroxy derivatives of AA (all derived from platelet 12-hydroperoxy-ETE) were generously provided by Dr. Robert Bryant (Wash. DC) (4, 5). LTB_4 was prepared from murine mastocytoma cells (6) and purified (7) as previously described. Epoxides and a diol derived from AA by cytochrome P450-dependent monooxygenase action (8–10) were a kind gift of Dr. J. Capdevila and Dr. N. Chacos (Dallas, TX). All compounds were diluted as stock solutions in ethanol with further dilutions in culture medium except for bradykinin (which was diluted directly in medium) and glucagon or BW755c (which were diluted in water). Control plates or tubes always contained identical amounts and types of diluent as in the experimental plates.

Isolation of islet cells. For studies of insulin release, monolayer cultures of dispersed neonatal rat pancreatic cells, enriched in the endocrine cell component, were established as previously described (2). These cultures contain 50% endocrine cells, the rest appearing to be typical fibroblasts. Of the endocrine cell population, 60% are beta cells, 20% alpha cells, and 10% each D and F cells. After being maintained in culture for 2 d in NCTC 135/TC199 (45%:45%) medium containing 300 mg/dl of glucose and 10% fetal calf serum, the cells were preincubated for 2 h in Eagle's minimal essential medium containing 100 mg/dl of glucose (to allow regranulation of cells) and 0.5% fetal calf serum. This was followed by a 1-h static incubation in fresh medium containing 300 mg/dl of glucose (except where otherwise indicated) and 0.5% serum. Medium from that 1-h incubation was collected for subsequent measurement of insulin and PGE. These incubation procedures have previously been described in detail (2). Test agents such as AA were generally present only during the final hour of incubation; where pretreatment of the cells was required, the agents were generally present during both the preincubation and incubation periods, except as indicated.

For studies of the metabolism of AA by the cells, a modification of this method was used. 20–30 pancreases from 2–3-d-old Sprague–Dawley rats were enzymatically digested using trypsin-collagenase as described (2). The dispersed cells were plated into tissue culture dishes and incubated in NCTC 135/TC medium 199 with 10% fetal calf serum. Following overnight incubation of the cells, the culture medium containing nonattached cells was collected and dishes were gently rinsed with culture medium to remove additional nonattached cells. This islet cell-enriched fraction was collected by centrifugation of the combined media, rather than being replated for monolayer culture. The cell pellet was used for cell labeling with [^{3}H]AA in the following manner. The cells were resuspended in 8–12 ml of calcium-free medium (Eagle's minimal essential medium with glucose 50 mg/dl) in the absence of any added proteins or serum, aliquoted into 2–4 plastic test tubes, and incubated with 2 μCi/ml of 5,6,8,9,11,12,14,15[^{3}H]AA (60–80 Ci/mmol; New England Nuclear, Boston, MA) at 37°C in 5% CO_2 and 95% air. During incubation, cells were frequently and gently mixed. In all studies, an incubation of control medium (without cells) was performed in an identical fashion to assess possible nonenzymatic autooxidation of AA. After 120 min, the medium was removed to determine the amount of radioactive AA ("exogenous AA") converted to its metabolites. Free AA was removed by washing the cells and test tube with culture medium containing 0.2% delipidated bovine serum albumin. This was followed by a 30-min incubation during which the membrane lipids into which the [^{3}H]AA had (presumptively) been esterified were deacylated using either the calcium ionophore A23187 (15 μM) or bradykinin (2.5 μg/ml) in serum-free medium containing 300 mg/dl of glucose and 2mM Ca^{++}. At the end of this period, the medium was collected and frozen for future determination of the metabolites

traenoic acid; HPLC, high performance liquid chromatography; hydroperoxy-ETE, hydroperoxyeicosatetraenoic acid; LTA_4, LTB_4, and LTC_4, leukotriene A_4, B_4, and C_4; MS, mass spectrometry; m/z, mass/charge; NDGA, nordihydroguaiaretic acid; PG, prostaglandin; t_r, retention time.

produced from AA released from lipid esters ("endogenous AA").

Intact rat islets were obtained from 250 to 300-g Sprague-Dawley rats by a modification of the method of Lacy and Kostianovsky (11) using a Ficoll gradient (12). From 100 to 500 islets were hand picked into plastic test tubes and treated similarly to the dispersed cells. (Additional studies were also performed in which intact rat islets were incubated in 1 ml Krebs Ringer-bicarbonate buffer, gassed in 95% O_2, 5% CO_2, pH 7.4, with or without 5–10% fetal calf serum or 0.4% bovine serum albumin. This protocol yielded similar results as studies using culture medium).

HPLC. Metabolites of AA in the medium were extracted by the method of Powell (13). This method involves selective retention of PG and HETE from medium applied to an octadecasilyl (C_{18}) minicolumn (Sep-Pak, Waters Associates, Milford, MA), followed by a wash with 15% methanol in water (10 ml) and petroleum ether (8 ml). Compounds of interest were then eluted using methyl formate or methanol. By adding known amounts of tritiated standards to culture medium, representative recoveries for various compounds from this extraction procedure were estimated to be: 6 keto-$PGF_{1\alpha}$, 88%; PGE_2, 91%; 15-keto, 13,14 dihydro-PGE_2, 93%; 12-HETE, 66%. The methyl formate was concentrated by evaporation in 6 × 50-mm test tubes and the test tubes were rinsed with acetonitrile (ACN), bringing the volume to 40 μl. An aliquot (10 μl) was then injected onto a Varian 5000 liquid chromatograph equipped with a free fatty acid column (Waters Associates) (system A). The flow rate of the mobile phase (23% ACN, 76.7% water, 0.3% acetic acid) was 2 ml/min for the first 50 min except for a 10-min period when the flow was reduced to 0.8 ml/min in order to separate PGE_2 from PGD_2, which otherwise coelute. At 50 min, the ACN concentration was increased from 23 to 80% in a linear gradient over the ensuing 30 min. Aliquots were collected every minute and counted by standard liquid scintillation spectrometric techniques. Neither BW755c nor NDGA altered the recovery of 12-HETE in the extraction or chromatographic steps.

To confirm further the identity of unknown compounds, samples containing the compounds of interest were analyzed using three additional chromatographic systems. 20–50 μl were injected onto an Altex 5-μ octadecasilyl column (25 cm length × 0.46-cm i.d., Altex Scientific, Inc., Berkeley, CA) developed isocratically at either 43 or 46% ACN in aqueous phosphoric acid (q.s. to achieve pH 2.3) at a flow rate of 1.75 or 1 ml/min, respectively (system B). This technique (using the higher ACN concentration) allows base-line separation of 15-HETE from 11-HETE, and 9-HETE from 5-HETE; 8-HETE and 12-HETE are only partially resolved from each other (R_s = 0.8). Elution times for the 15-, 11-, 8-, 12-, 9-, and 5-HETE isomers are: 84–87, 95–99, 103–106, 105–109, 112–116, and 118–122 min, respectively. After it was ascertained that no UV-detectable 9-HETE was present in the sample, it was included as an internal standard. The pure HETE standards were generously provided by Dr. Robert Bryant (George Washington University, Wash. DC). In addition, samples were analyzed using the same column but a different mobile phase (76% methanol; 24% water with phosphoric acid; q.s. pH 2.7; 1 ml/min: system C). The peak retention times (t_r) of the 6 monohydroxyeicosatetraenoic acid isomers are shown in Fig. 3. The same samples were also analyzed using 70 and 73% methanol in the mobile phase (0.5 or 1 ml/min).

The fractions containing the hydroxy-fatty acids (in their free acid form) were then collected, methylated in ether using diazomethane and analyzed by HPLC. The octadecasilyl column was again used, but with an ACN concentration of 73% and a flow rate of 1.5 ml/min (system D). Using this system, 15- and 11-HETE-methyl esters are totally resolved, as are 11- and 12-HETE-ME ($R_s > 1.25$); 12- and 8-HETE-ME are only partially resolved ($R_s \sim 0.7$), as are 9- and 5-HETE-ME ($R_s \sim 0.7$). Retention times of peaks are: 10.8, 11.9, 12.2, 12.6, 13.1, and 13.4 min, respectively, for the methyl esters of 15-, 11-, 12-, 8-, 9-, and 5-HETE.

Gas chromatography-mass spectrometry (GC-MS). For mass spectrometric studies, a pooled sample of medium was chromatographed using system 3, and the peak putatively identified as 12-HETE (based on the t_r of the peak of tritium counts) was isolated. Methanol was removed under a nitrogen stream and the volume was brought to 1 ml with distilled water. The sample was extracted with 2 ml of chloroform; the $CHCl_3$ layer was washed with 1 ml of water and dried over sodium sulfate. The organic layer was transferred to a conical derivatization vial, evaporated to dryness, solubilized in 50 μl of methanol, and 250 μl of ethereal diazomethane was added. After 10 min at room temperature, the sample was evaporated to dryness and 50 μl of ACN and 50 μl of *bis*(trimethylsilyl) trifluoroacetamide (BSTFA, Supelco, Inc., Bellefonte, PA) were added sequentially. After 20 min at 60°C, the sample was cooled and the BSTFA and ACN were removed under a nitrogen stream. The sample was totally loaded (in 5 μl of hexane) onto the glass needle top of a falling needle injector installed on a Hewlett-Packard 7625a gas chromatograph (Hewlett-Packard Co., Palo Alto, CA). The GC column used was a 30-m flexible fused silica column (i.d.: 0.25 mm, phase SE-30 at 0.25 μm film thickness; J & W Scientific Co., Rancho Cordova, CA). The column outlet was interfaced directly to the ion source of a VG Micromass 15 mass spectrometer (VG Micromass, Ltd., Altrin-cham, U.K.). Data collection was controlled by a VG 2050 data system. The carrier gas was helium, with flow rate of 1.5 ml/min. The mass spectrometer was operated in the electron impact mode (70 eV), with a source temperature of 180°C and 4 kV accelerating potential. Selected ion monitoring was accomplished by varying the accelerating potential in order to monitor the ratios of mass/charge (m/z) 229: m/z 295. Results for the unknown sample were compared with those using low nanogram quantities of authentic 12-HETE.

Insulin radioimmunoassay. Insulin (14, 15) and PGE (2, 3) values were estimated by standard radioimmunoassay techniques, as previously described.

Statistical analysis. Comparisons were made using the Newman-Keuls test for multiple comparisons and the nonpaired *t* test for isolated comparisons. Unless otherwise stated, comparisons were made on data obtained from groups of four plates.

RESULTS

Identification of AA metabolites. Three cyclooxygenase products of the dispersed neonatal pancreatic cells were consistently identified by HPLC (system A): PGE_2, $PGF_{2\alpha}$, and 6-keto $PGF_{1\alpha}$, the hydrolytic metabolite of prostacyclin (Fig. 1). Production of the PG was found both in direct labeling studies and in studies wherein the release of incorporated AA was stimulated. Identical chromatograms were seen following incubation of isolated intact rat islets. Production of PG was reduced by indomethacin (10 μg/ml; Fig. 1) as well as by sodium salicylate, 20 or 100 mg/dl (data

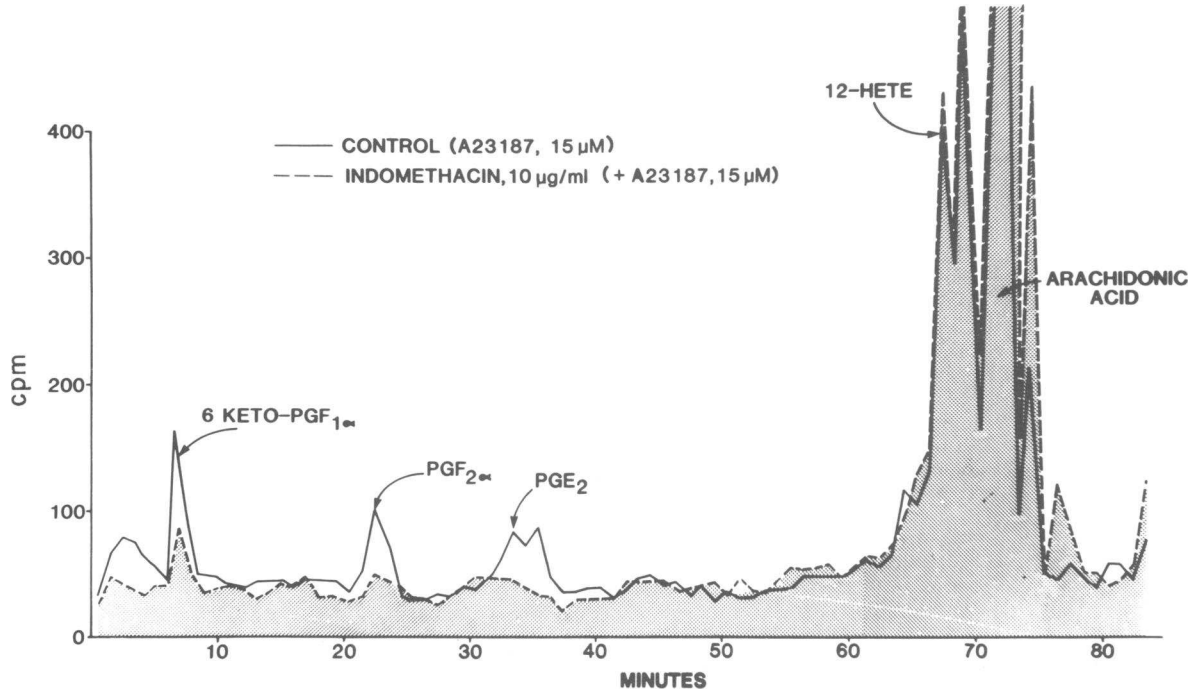

FIGURE 1 Radiochromatogram of products of [^{3}H]AA released from neonatal dispersed islet cell lipids by the divalent ionophore A23187, in the presence and absence of indomethacin, a cyclooxygenase inhibitor. HPLC system A was used. Prostaglandins and 12-HETE comigrated precisely with their corresponding tritiated PG and 12-HETE standards. Hydroperoxy-ETE elute 25–30 s after HETE in this system and therefore cannot be well differentiated from the HETE peak using 1-min sampling for counts per minute. Standards for thromboxane B_2 elute at 16–20 min; for PGE_1, 39–40 min; for 15 keto,13,14 dihydro PGE_2 at 45–47 min; for PGA_1, at 58–60 min; for LTB_4 and LTC_4 at 61–62.5 min; and for HETE-methyl esters at 70–72 min. Preliminary studies showed that the epoxides of AA (epoxy-eicosatrienoic acids) formed by monooxygenase action (9) elute between 68 min and AA. Epoxy-eicosatetraenoic acid derivatives formed by lipoxygenase would be expected to elute slightly earlier than epoxy-eicosatrienoic acids, due to an additional double bond. The peak eluting after 12-HETE is unidentified but is likely an epoxy- or keto-derivative of AA as judged by published reverse-phase elution patterns (9, 16, 17). The small peaks at 51–57 and 63–66 min (which were more prominent in other studies) coelute with isomeric mixtures of trihydroxyeicosatrienoic acids (4, 5, 19) and hydroxy-epoxy-eicosatrienoic acids (HEPA) (4, 5), respectively. The presence of HEPA was confirmed as well using HPLC system C. The small peaks at 42–48 min were variably prominent in other studies. Although they migrate near 15 keto, 13,14 dihydro PGE_2 and the corresponding metabolite of $PGF_{2\alpha}$, their relative insensitivity to inhibition by BW755c, as well as their broad appearance and chromatographic mobility, suggest that they may be isomeric, polyoxygenated degradation products of 12-hydroperoxy-ETE.

not shown) and low concentrations of BW755c (Fig. 2). In isolated instances only, a small peak eluting with or near PGD_2 or thromboxane B_2 was observed. In most studies, a peak (or peaks) eluting at 41–48 min in the region expected for the 15-keto-13,14-dihydro metabolites of PGE_2 and $PGF_{2\alpha}$ was also observed. However, although definite identification of these peaks was not attempted, it is more likely that these represent polyoxygenated lipoxygenase products (see below and legends to Figs. 1 and 2).

In addition, in studies both of intact islets and of dispersed pancreatic cells, at least two larger peaks were observed, one comigrating with authentic 12-HETE standard and one eluting between 12-HETE and AA (Fig. 1). The later peak is not firmly identified. However, its elution position corresponds in general to the published chromatographic behavior of an epoxide of AA (9, 16) or the keto degradative product of hydroperoxy-ETE (17). It therefore is provisionally identified as a lipoxygenase intermediate. Neither peak was reduced by indomethacin (Fig. 1). Synthesis of the compound comigrating with 12-HETE standard was reduced by coincubation with NDGA (−89% at 50 μM) or BW755c (−54% at 250 μM, Fig. 2).

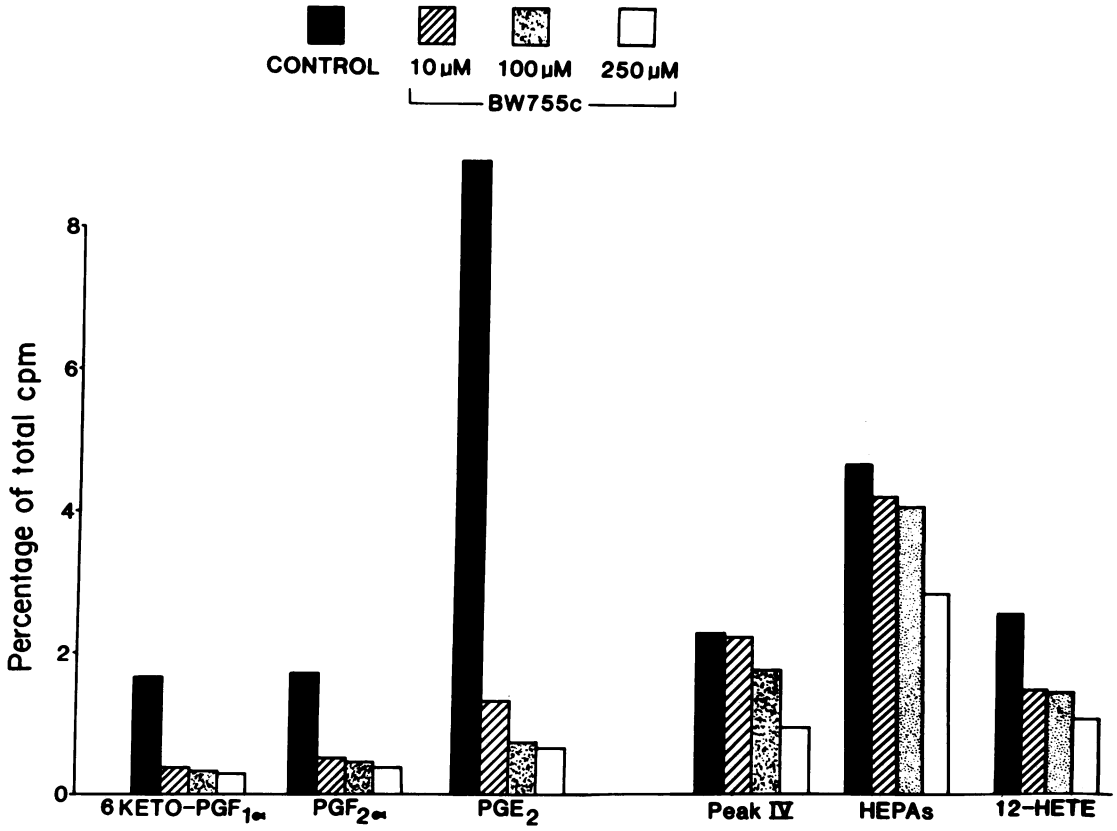

FIGURE 2 Differential sensitivities of three cyclooxygenase products (6 keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, PGE_2), 12-HETE, and two other putative lipoxygenase products ("peak IV" and hydroxyepoxyeicosatrienoic acids or "HEPA") to inhibition by BW755c. Results are expressed as a percentage of total counts per minute eluting as defined peaks from the HPLC column using system A. Cyclooxygenase products are potently inhibited at 10 μM of BW755c; lipoxygenase products were relatively resistent to inhibition at low drug concentrations. Similar results were seen using NDGA. Peak IV is presumably an unidentified polar lipoxygenase product eluting at 42–48 min in Fig. 1.

In the primary HPLC used (system A: free fatty acid column), the six HETE isomers are poorly, or not at all, separated. Therefore, samples were also analyzed using HPLC system B (5-μm octadecasilyl column; 46 or 43% ACN) and HPLC system C (same column; isocratic elution in 70, 73, or 76% MeOH at 0.5 or 1 ml/min). A predominant peak coeluting with authentic unlabeled 12-HETE was seen in all systems under all conditions described (cf. Fig. 3). Much smaller amounts of other HETE (especially 15-HETE) appeared to be made *de novo* in occasional studies but no HETE other than 12-HETE was consistently observed. 12-HETE was not detectable in control studies and therefore its presence was due to *de novo* synthesis. In contrast, 5-, 15- and especially 11-HETE were the major HETE in the autooxidation control samples.

After methylation of the fractions eluting in the regions expected for 12-HETE, a clear peak of radioactivity was seen to coelute with authentic 12-HETE-methyl ester (system D).

Finally, additional confirmation that the major peak eluting with 12-HETE on HPLC was indeed that compound was provided by GC-MS based on the following data: (*a*) coelution (with an authentic, derivatized sample of 12-HETE) of the methyl ester-trimethylsilyl ether derivative of the unknown peak on a 30-m SE-30 capillary column (t_r = 4 min, 46 s); (*b*) presence of the characteristic saddle effect seen in the gas chromatogram of HETE (18); (*c*) a major fragment ion at m/z 295, with an ion ratio of m/z 229: m/z 295 of 0.12, characteristic of 12-HETE.

In some studies (using the free fatty acid column; system A) the shoulder at 63–66 min (Fig. 1) was seen as a larger and well-delineated peak which also was reduced by high concentrations of BW755c (−41% at 250 μM; "HEPA", Fig. 2) or NDGA (−70% at 50 μM),

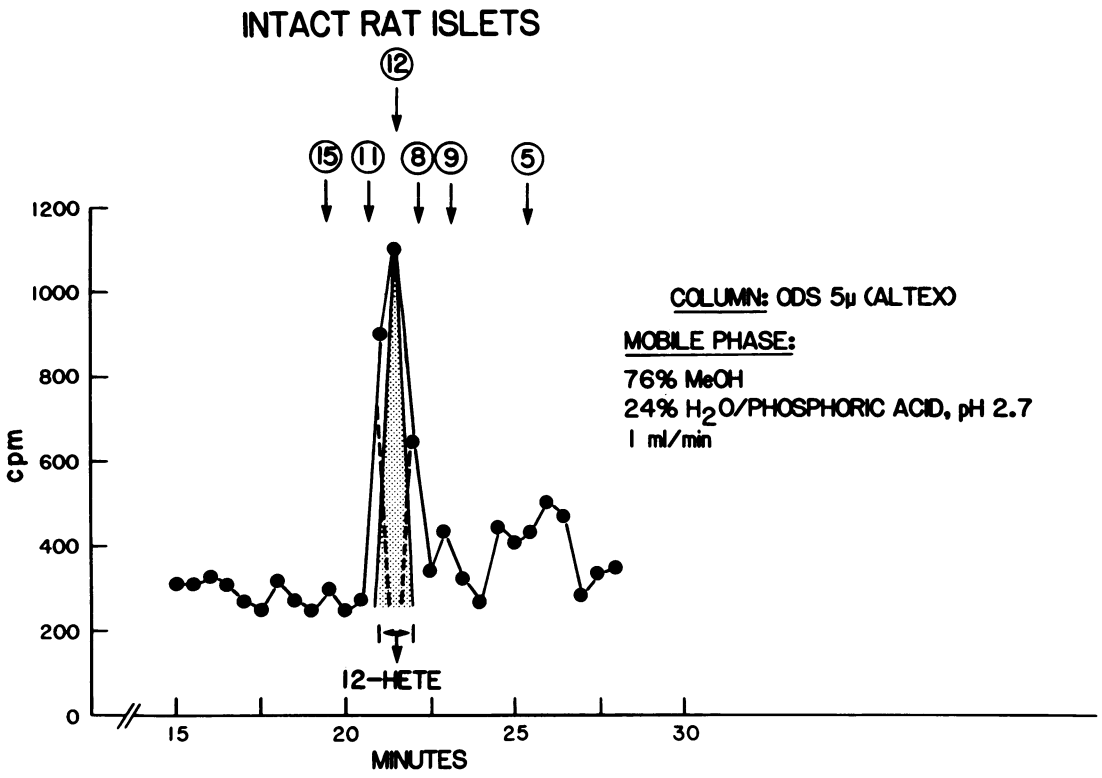

FIGURE 3 Chromatogram of products synthesized by intact rat islets incubated in Krebs-Ringer-bicarbonate buffer. System C was used, with a flow rate of 1 ml/min and a MeOH concentration of 76%. Fractions were collected every 30 s and counted by scintillation spectrometry. Numbers above chromatograms refer to elution peaks of unlabeled HETE isomer standards migrating at the indicated locations in the same run. Dashed lines (for 11- and 8-HETE) and stippled peak (for 12-HETE) indicate the extent of UV-detectable peaks seen with each HETE (unlabeled) injected individually as a standard under the same conditions. A major peak migrating with 12-HETE is seen; no detectable 12-HETE was found in the control incubation. In contrast, the smaller peaks eluting with 15-, 9- or 5-HETE and the larger 11-HETE peak (seen as a shoulder on the main peak) are fully explicable (based on control studies) by HETE produced by autooxidation or contaminating the commercially obtained [^{3}H]AA. Identical results were seen using 73 or 70% MeOH in the mobile phase.

but unlike cyclooxygenase products was poorly inhibitable at lower concentrations (Fig. 2). This peak comigrates on this HPLC system as well as on system C with an isomeric mixture of the hydroxy, 11,12-epoxy derivatives that are degradation products of 12-hydroperoxy-ETE (5). The small peak at 51–57 min (Fig. 1) co-elutes with an isomeric mixture of trihydroxyeicosatrienoic acids, which are also nonenzymatically formed breakdown products of 12-hydroperoxy-ETE (4, 5, 17, 19). Similarly, in most studies an additional peak(s) was seen at 41–48 min (see above); this peak was less well inhibited by BW755c than cyclooxygenase products (Fig. 2; peak IV). The identity of this peak is uncertain but it is presumed to represent a very polar, polyoxygenated lipoxygenase metabolite.

In each of six studies, a major contribution of nonenzymatic formation of 12-HETE by autooxidation (or contamination with 12-HETE of the [^{3}H]AA used) was excluded by the observation that the percentage of total counts recovered from the column in the 12-HETE region was always greater (6.43±1.23%; $P < 0.01$) when cells were present than when identical incubations and extractions were performed using [^{3}H]AA in the absence of cells (2.07±0.4%). These results were found whether intact islets or dispersed cell preparations were used. As indicated above, this presence of HETE in control studies could be accounted for by autooxidative formation of, or contamination of the [^{3}H]AA used with, the 5-, 15-, and especially the 11-isomer of HETE, with lesser amounts of 8- and 9-HETE.

Insulin responses to endogenous and exogenous AA and its metabolites. When monolayer cultures of

neonatal rat pancreatic cells were incubated for 1 h with AA (5 μg/ml; 16 μM) in 300 mg/dl glucose, insulin release was potently augmented (Figs. 4 and 5). This stimulation of insulin release was dose dependent (Fig. 4) and at no concentration of AA (between 50 ng/ml and 25 μg/ml) did inhibition of insulin release occur. The AA effect was also time dependent (Fig. 5), since the insulin released during 1 h of incubation was much greater if it was preceded by a 3-h preincubation period in AA (5 μg/ml). The AA effect also was reversible (Fig. 5), since cells from which the exogenous AA had been washed after a 3-h preincubation period in AA had a rate of insulin release no different from cells never exposed to exogenous AA (Fig. 5). The insulinogenic effect of AA (5 μg/ml) did not appear to be due to nonspecific or toxic effects, since this concentration of AA had no effect upon cell morphology (as assessed by phase contrast microscopy) or upon cell viability (as assessed by trypan blue exclusion). Higher concentrations of AA (≥25 μg/ml) did cause some rounding of cells as well as some cell detachment or lysis. However, this effect appeared to be preferentially directed towards the fibroblasts also present in these cultures. Nonetheless, all other studies involving insulin secretion used a submaximally effective concentration of AA (5 μg/ml) that caused no morphologic changes.

The cyclooxygenase inhibitor, indomethacin (0.5 μg/ml), greatly potentiated the stimulatory effect of AA (5 μg/ml) (Fig. 6), in contrast to the weak (though significant) stimulation induced by indomethacin in the absence of exogenous AA. Sodium salicylate (20 mg/dl), another inhibitor of PG synthesis in this system (2), also potentiated the stimulation of insulin release by AA (data not shown). In contrast, the effect of AA was totally abolished by a combined 2-h pretreatment and 1-h incubation with either of two structurally dissimilar, combined lipoxygenase and cyclooxygenase inhibitors, BW755c and ETYA, the acetylenic analogue of AA (Fig. 7).

To examine the specificity of the AA effect, other long chain fatty acids of varying degrees of unsaturation were studied at 300 mg/dl of glucose. Two saturated fatty acids of similar chain length, stearic acid and arachidic acid (5 μg/ml), had no effect on insulin release. The monoene oleic acid (5 μg/ml) did stim-

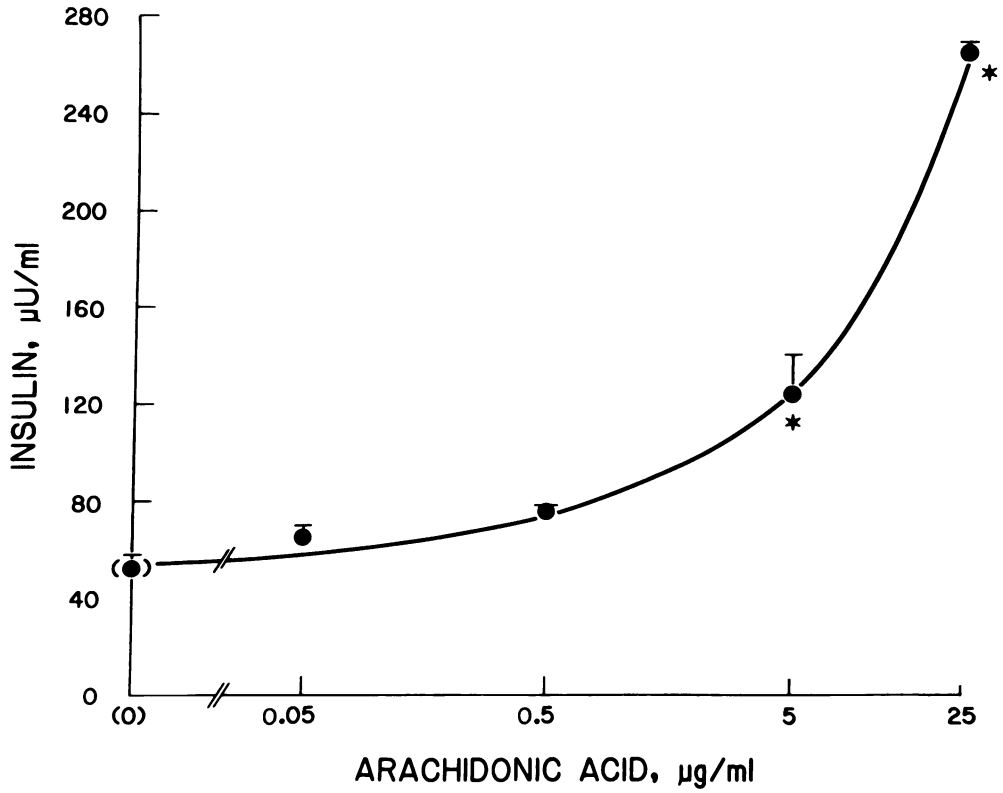

FIGURE 4 Effect of varying concentrations of AA on insulin release in the presence of glucose, 300 mg/dl, over a static, 1-h incubation period. Data represent mean ± SEM, n = 4 plates each.
*$P < 0.001$.

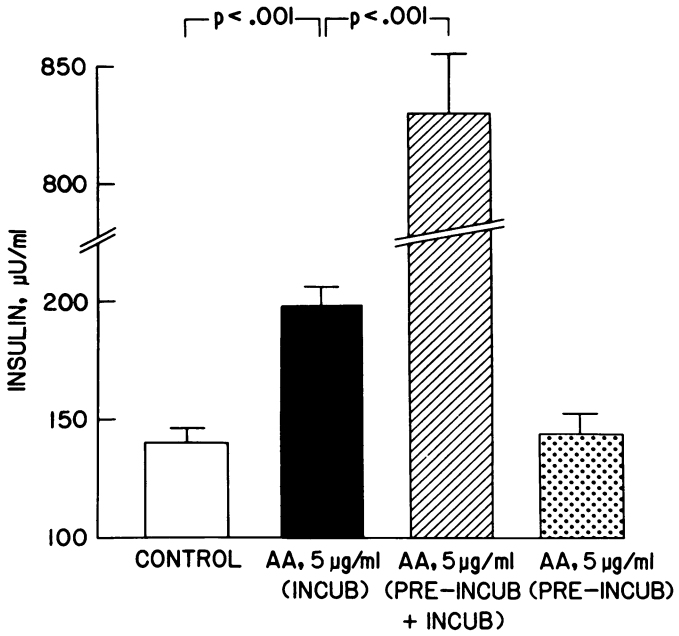

FIGURE 5 The time-dependence and reversibility of the effect of one concentration (5 μg/ml) of AA to stimulate insulin release. Arachidonate was present (*a*) for a 1-h incubation only (second panel); (*b*) for a 3-h preincubation period (followed by a wash and medium change) and a 1-h incubation period (third panel); (*c*) for a 3-h preincubation period only, after which AA was washed from the plates. Insulin release is measured over the last 1-h incubation period when the glucose concentration was 300 mg/dl. AA stimulation of insulin release can be seen to be time dependent (being greater when present for 4 h than for 1 h), and reversible (since insulin release is similar whether AA is never added or is added for 3 h and then washed from the plates). Data represent mean±SEM, n = 4 plates each.

ulate insulin release (control: 46±2; oleic acid: 295±9 μU/ml; mean±SEM; n = 4 plates each; $P < 0.001$); however, this effect was only partially blocked (−36%) by BW755c (193±3 μU/ml; $P < 0.001$ vs. oleic acid alone).

The stimulation of insulin release by glucose could be largely prevented by either of three lipoxygenase inhibitors. For example, the stimulation of insulin release by glucose (glucose, 30 mg/dl: 33±2; glucose, 300 mg/dl: 59±4 μU/ml; $P < 0.01$) was totally abrogated by BW755c, 250 μM (36±2 μU/ml). Similar data were seen with NDGA (glucose, 30 mg/dl; 40±3; glucose, 300 mg/dl: 70±4, $P < 0.01$; glucose, 300 mg/dl + NDGA, 20 μM: 38±2 μU/ml) or ETYA (glucose, 30 mg/dl: 45±2; glucose, 300 mg/dl: 99±2, $P < 0.01$; glucose, 300 mg/dl + ETYA, 25 μM: 72±1 μU/ml, $P < 0.01$ vs. glucose, 300 mg/dl alone). Similarly, the effect of glucagon, another physiologic insulin secretagogue, was considerably reduced (glucose 300 mg/dl: 48±2; glucose, 300 mg/dl + glucagon, 250 ng/ml; 151±7, $P < 0.01$; glucose, 300 mg/dl + glucagon + BW755c, 250 μM: 69±5 μU/ml, $P < 0.01$ vs. glucose, 300 mg/dl and glucagon). Two structurally dissimilar putative activators of phospholipase A_2 or C (bradykinin; divalent cation ionophore A23187) were studied to increase the release of endogenous AA. AA release was assumed to be reflected in increments in PGE synthesis (Table I). Although the lowest concentration (1 μg/ml) of bradykinin slightly stimulated insulin release by itself (Table I, experiment 1), at higher concentrations (5–10 μg/ml) this effect was evident only when PG synthesis was inhibited by indomethacin (Table I, experiments 2 and 3). Thus, indomethacin unmasked a stimulation of insulin release by bradykinin when the cyclooxygenase pathway was inhibited (Table I, experiments 2 and 3: cf. indomethacin vs. indomethacin + bradykinin). The stimulatory effect was blunted, but not totally abrogated, by lipoxygenase inhibition, despite an additional inhibition of cyclooxygenase when BW755c was added to indomethacin (Table I, experiment 3).

The divalent ionophore A23187 (0.25 to 5 μg/ml) stimulated insulin release, an effect potentiated by indomethacin (Table I, and below). The effect of a high concentration of A23187 (5 μg/ml) was blunted by NDGA, 20 μM (Table I). However, this blockade was only partial. Furthermore, no blockade by BW755c was seen at lower concentrations of ionophore (control

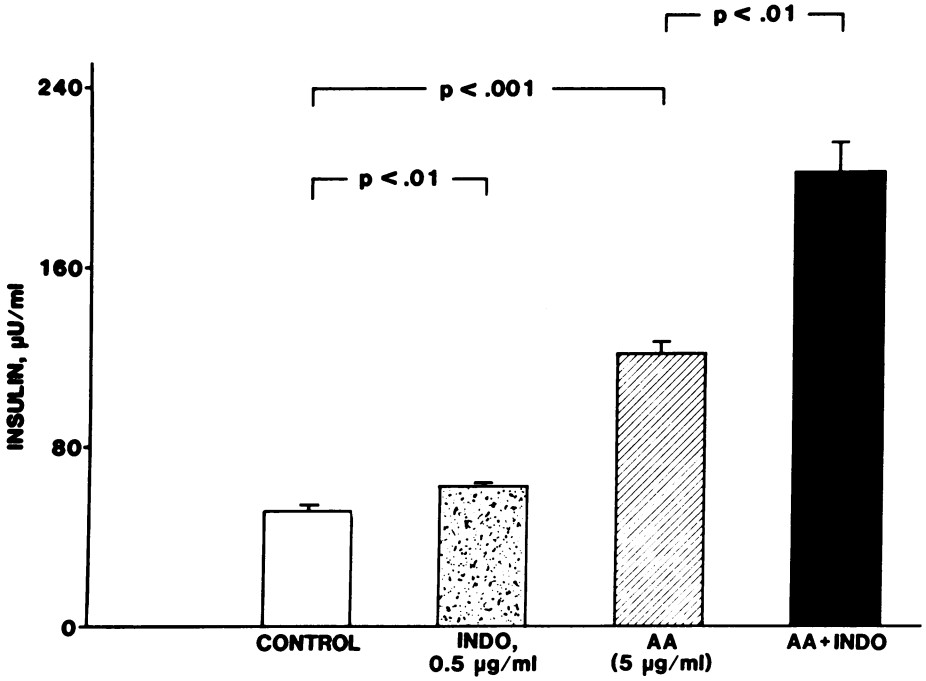

FIGURE 6 Effect of indomethacin (INDO) and AA, alone and in combination, on insulin release at 300 mg/dl of glucose, during a 1-h incubation period. Data represent mean±SEM, $n = 4$ plates each.

16±1; A23187, 0.25 μg/ml; 55±1, $P < 0.001$; BW755c + A23187, 53±2 μU/ml, P = NS vs. A23187 alone). At this low concentration, A23187 appeared not to potently activate phospholipase, since PGE levels did not rise compared with control (control: 280±19; A23187: 223±16 pg/ml).

As indicated in Table II, 12-HETE (up to 1 μg/ml) had no effect on insulin release at 300 mg/dl of glucose. 12-Hydroperoxy-ETE augmented release with a peak effect at 200 ng/ml. Since 12-HPETE is highly labile and also may not reach intracellular sites (for further metabolism) well when given exogenously,

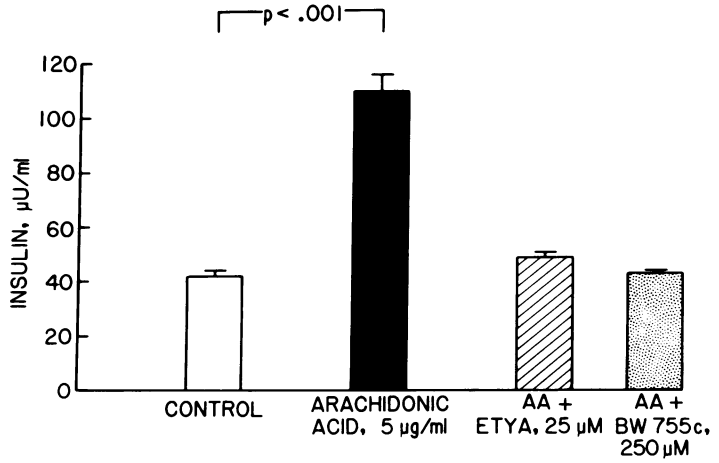

FIGURE 7 Prevention by lipoxygenase inhibitors (ETYA or BW755c) of AA-induced insulin release at a stimulatory glucose concentration (300 mg/dl). The cells were preincubated in ETYA or BW755c for 2 h before addition of AA for a final 1-h incubation period in the continued presence of the lipoxygenase inhibitor. Data represent mean±SEM, $n = 4$ plates each.

TABLE I

Effects of Putative Phospholipase Activators and of Inhibitors of AA Metabolism on Insulin and PGE Release

	Insulin		PGE	
	μU/ml		pg/ml	
A. Divalent ionophore A23187				
Control (ETOH)	101±3		628±112	
A23187, 1 μg/ml	279±5*		2,132±137*	
A23187, 5 μg/ml	334±4*	$P < 0.001$	2,453±174*	$P < 0.001$
A23187, 5 μg/ml + indomethacin, 5 μg/ml	370±10*		611±188	
A23187, 5 μg/ml + NDGA, 20 μM	225±3		1,620±192	
Indomethacin alone	130±3‡		388±199	
NDGA alone	71±5*		399±85	
B. Bradykinin				
Exp. 1				
Control (H_2O)	66±1		505±26	
Bradykinin, 1 μg/ml	75±1		1,513±113*	
Exp. 2				
Control (H_2O)	63±3		937±98	
Bradykinin, 5 μg/ml	59±1	$P < 0.001$	1,966±304‡	$P < 0.001$
Bradykinin + indomethacin, 5 μg/ml	90±1*	$P < 0.01$	643±32	
Indomethacin, 5 μg/ml	68±3		—	
Exp. 3				
Indomethacin, 5 μg/ml	45±1		—	
Indomethacin, 5 μg/ml + bradykinin, 10 μg/ml	62±1*	$P < 0.001$	562±65	$P < 0.001$
Indomethacin + bradykinin + BW755c, 250 μM	40±0.25		119±7	
BW755c, 250 μM alone	30±1		83±18	

Glucose = 300 mg/dl. All values represent PGE or insulin accumulation over a 1-h static incubation. n = 3–4 plates each (insulin); 2–4 plates each (PGE).

* $P < 0.001$ vs control (Condition No. 1 in each experiment).

‡ $P < 0.005$–0.01.

diethylmaleate was then studied as a means to deplete intracellular reduced glutathione and shunt AA as substrate from glutathione-dependent derivatives (HETE, LTC_4, other glutathionyl derivatives of fatty acid epoxides) to nonglutathione-requiring derivatives (hydroperoxy-ETE, LTA_4, other fatty acid epoxides) (20). Diethylmaleate alone stimulated insulin release. It also markedly potentiated the stimulatory response to AA (Table II). Furthermore, 3 epoxy-derivatives of AA derived via monooxygenase action (8–10), which are LTA_4 analogues, all stimulated insulin release, whereas a corresponding vicinal diol was without effect (Table II). Correspondingly, the diol derived from LTA_4 (LTB_4, 2–160 ng/ml) did not stimulate insulin release; however these latter studies must be considered preliminary, as the concentrations of LTB_4 available for use were very limited.

DISCUSSION

These data document, for the first time, both a dependence of hormone secretion on the integrity of the lipoxygenase pathway and a specific role for lipoxygenase metabolites in endocrine function. Our studies suggest that a lipoxygenase that converts AA to a hydroxy fatty acid, 12-HETE (as well to other, more polar, lipoxygenase products), is present in islet endocrine cells. The identification of 12-HETE was based on retention times of the compound precisely matching those of either cold or tritiated 12-HETE and 12-HETE methyl ester standards in four separate HPLC systems under eight different chromatographic conditions. The identity as 12-HETE was further supported by the gas chromatographic and mass spectroscopic features of the unknown peak. The presence of a lipoxygenase in this preparation is further supported by the inhibition of 12-HETE formation by either BW755c or NDGA; the concentrations required to maximally reduce 12-HETE formation were greater than those required to inhibit production of cyclooxygenase products. Lipoxygenase products were found both after direct exposure of cells to exogenous [^{3}H]AA and after release of endogenous (esterified) substrate. It is possible that the HETE measured is a minimal figure, since HETE may be reincorporated to some degree into cells (21); indeed our preliminary studies show that additional HETE (but not PG) may be found after sonicating the cells. An important contribution

TABLE II

Effect of 12-HETE, 12-Hydroperoxy-ETE, AA, Diethylmaleate (DEM), and Monooxygenase-derived Epoxide Derivatives of AA on Glucose-induced Insulin Release

Condition		
Control		37±2
12-HETE, 5 ng/ml		40±2
12-HETE, 500 ng/ml		40±3
12-HETE, 1 μg/ml		46±1
Control		97±1
12-Hydroperoxy-ETE, 0.05 μg/ml		120±1*
12-Hydroperoxy-ETE, 0.2 μg/ml		126±2*
Control		62±2
DEM, 1 mM		103±3*
AA, 5 μg/ml	$P < 0.001$	273±4*
DEM + AA		441±8*
Control		107±1
5,6-Epoxy, 8,11,14-eicosatrienoic acid (ETA), 400 ng/ml		143±1*
8,9-Epoxy-5,11,14-ETA, 400 ng/ml		135±7‡
14,15-Epoxy-5,8,11-ETA, 400 ng/ml		121±4§
5,6-Dihydroxy-8,11,14 ETA, 400 ng/ml		109±3

Data represent mean±SEM, n = 4–8 plates each; all values at 300 mg/dl glucose.

* $P < 0.001$ vs. control.

‡ $P < 0.005$.

§ $P < 0.025$–0.01.

of autooxidation to the measured 12-HETE production was excluded by appropriate control experiments and by the inhibition of formation by lipoxygenase blockers. Smaller amounts of other HETE were found in occasional studies but no HETE other than 12-HETE was consistently synthesized. Nonetheless, enzymatic formation of very small amounts of HETE isomers other than 12-HETE cannot be absolutely excluded especially if they could be derived from a (hypothetical) compartmentalized pool of arachidonate not mobilized during the procedures used in the current study. The presence of lipoxygenase products implies that a lipoxygenase, or a similar oxidative enzyme, is present in islet endocrine cells and that the unstable intermediate (12-hydroxyperoxy-ETE) is also formed. The latter conclusion is supported by the presence of several other peaks coeluting with degradation products of 12-hydroperoxy-ETE (17): epoxy-hydroxy-eicosatrienoic acids, trihydroxy-eicosatrienoic acids, and possibly 12-keto-AA. Although HETE may be formed by nonlipoxygenase oxidative pathways such as a cytochrome P450-dependent monooxygenase (8–10) or an oxygen radical-xanthine oxidase pathway (22), the inhibition of insulin release and blockade of the formation of these compounds by lipoxygenase inhibitors argues against these as the dominant sources of HETE formation in our system. Kelly and Laychock (23) have also observed production by intact rat islets of a compound migrating chromatographically with an unidentified HETE; production of this compound was inhibited by NDGA (24). Since in our studies both endocrine cell-enriched, dispersed neonatal rat pancreatic cells and intact rat islets synthesized 12-HETE, it appears likely that the islet endocrine cells were responsible for the majority of the 12-HETE formation. The overnight culture of the cells and the lack of thromboxane synthesis argue against retained platelets as the source of the 12-HETE.

In addition we, as well as Kelly and Laychock (23, 24) have observed that islet endocrine cells synthesize PGE_2, $PGF_{2\alpha}$, and prostacyclin. Using either thin-layer chromatography or HPLC, these investigators also found compounds comigrating with the 15-keto,13,14 dihydro-metabolites of PGE_2 and $PGF_{2\alpha}$ in intact islets. Although we have observed a broad peak coeluting in or near the location expected for these metabolites, such an identification is tenuous. We previously found by radioimmunoassay that little or no 15-keto,13,14-dihydro-PGE_2 is formed by these cells (2). Furthermore, the concentration of BW755c required to inhibit synthesis of this peak was more compatible with its identity as a polyoxygenated lipoxygenase (rather than cyclooxygenase) product. The current observation identifying PGE_2 by HPLC as a product of pancreatic islet cells does lend further support to our radioimmunoassay data identifying PGE as a major product of islet endocrine cells (2) and (in conjunction with the potentiation by indomethacin or sodium salicylate of the insulinogenic response to AA) support the formulation that PGE (2, 25) and prostacyclin (26) may be physiologic negative modulators of glucose-induced insulin release. We did not find evidence for the production of substantial amounts of PGD_2 or thromboxane A_2 by islet endocrine cells. However, it should be recognized that the identification of AA metabolites produced by cellular systems is influenced not only by the enzymic capabilities of the cells studied, but also by the cofactors present (such as thiols) (27), the albumin concentration (28), and the substrate concentration as well as its source (endogenous vs. exogenous) (29–31). Thus, we cannot totally exclude production of other compounds by islet endocrine cells under different experimental circumstances.

Although both lipoxygenase and cyclooxygenase products were synthesized by either of the cell systems studied, addition of AA led to stimulation of insulin release. In fact, no concentration of AA between 50 ng/ml and 25 μg/ml inhibited insulin release. Although we have not demonstrated that increasing concentrations of exogenous AA lead to commensuratively

greater synthesis of specific lipoxygenase-derived metabolites, the AA effect was further augmented by inhibition of PG synthesis and was prevented by two structurally dissimilar lipoxygenase inhibitors (despite coinhibition of the cyclooxygenase cascade). These data suggested that products of the lipoxygenase pathway mediated AA stimulatory effect. Additionally, our preliminary data (32, and manuscript submitted for publication) suggest that BW755c, ETYA and a third lipoxygenase inhibitor, NDGA, inhibit cyclic AMP accumulation and glucose- and glucagon-induced insulin release in the absence of exogenously added AA. These findings support a physiologically important, specific role for lipoxygenase-mediated products of AA oxygenation as mediators of insulin secretion.

The insulin release induced by AA did not appear to be due to an undefined toxic effect on the cells since the effect was completely reversible upon removal of AA from the plates, was unaccompanied by evidence of cell injury at the primary AA concentration chosen for this study (5 μg/ml), and was pharmacologically inhibitable. Additionally, in the current studies in which AA was expected to be released from endogenous pools through activation of phospholipases by bradykinin (33) and calcium ionophore (34), stimulation of insulin release was also observed. In the case of bradykinin, stimulation was only convincingly seen in the presence of indomethacin, an observation that may be explained by concomitant stimulation of cyclooxygenase (35) as well as of phospholipase (33) activity by bradykinin. Others have also observed stimulation of insulin release by bradykinin (36), ionophore (37), and other drugs, such as furosemide (38) and tumor-promoting phorbol esters (39), which are felt to activate phospholipase(s). Recently it has been shown that exogenous phospholipase C closely mimics the effects of glucose to selectively increase phosphatidylinositol turnover and to augment insulin release (40), apparently by activating a phospholipid-dependent protein kinase C. Interestingly, this kinase was activated by diacylglycerols containing arachidonate,[2] linoleate, or oleate but not those containing stearate. It seems reasonable, therefore, to postulate that arachidonic acid release,[2] via its oxygenated metabolite(s), may be a common final pathway by which several membrane-active agents, including glucose, promote insulin release. In the current study, the stimulation of insulin release by glucose, glucagon, A23187 (high concentrations), and bradykinin was blunted by lipoxygenase inhibition. The relatively poor blockade of the effects of A23187 could be explained by incomplete inhibition of lipoxygenase, but it is also possible that direct alterations in ion flux (via provision of an exogenous ionophore) can bypass some specific consequence of lipoxygenase inhibition.

Comparable concentrations of arachidic acid or stearic acid, two saturated fatty acids of 20 and 18 carbon length, respectively, did not stimulate insulin release. However, another unsaturated fatty acid, oleic acid, did stimulate insulin release; its effect was only partially inhibited (−36%) by BW755c. Several other unsaturated fatty acids (linoleic, elaidic, palmitoleic, and α-linolenic acid) also stimulate insulin release in this system (unpublished results), an effect seemingly unrelated to relative chain lengths, degree of unsaturation or position of the omega double bond. The stimulatory effect of oleic acid and other fatty acids on insulin release has been known for many years (44, 45) and may simply relate in part to their use by the beta cell as fuels or the ability to alter intracellular levels of glucose metabolites (45). It is unlikely that these unsaturated fatty acids are directly lipoxygenated since several of them lack the substrate specificity required by lipoxygenase for compounds with a 1,4-pentadiene configuration (46). Substantial autooxidation of oleate is unlikely since this occurs only very slowly compared with arachidonate (47). However, unsaturated fats, such as oleic acid, can be converted to other oxygenated products such as epoxy- or dihydroxy-compounds (48) (which could alter insulin release) via alternative oxygenation systems such as a cytochrome P450-dependent oxygenase (8–10). Furthermore, polyunsaturated fatty acids can activate phospholipase (49, 50) and, after oxidation, some (e.g., 12-hydroxy-oleic acid or ricinoleic acid) activate lipoxygenase (51) while inhibiting prostaglandin synthetase (cyclooxygenase) (52). Indeed, the response to exogenous AA itself may be mediated not directly but by activation of endogenous AA release (30, 53). This formulation is in accord with the failure of saturated fatty acids (stearic or arachidic acids) to stimulate insulin release, which corresponds well to their inability to activate oleate- and arachidonate-sensitive phospholipase(s) (49, 50). Therefore, fatty acids (other than AA) in effect shunt AA into the lipoxygenase pathway by increasing substrate (AA) release but impeding PG synthesis. Thus, fatty acids could stimulate insulin release via both lipoxygenase-independent and lipoxygenase-dependent mechanisms. The distinction between a specific, lipoxygenase-dependent effect of AA and an additional, unspecific effect of other polyunsaturated fatty acids is supported by data describing

[2] This schema does not, of course, exclude an additional role for other products of phospholipase activation such as diacylglycerols themselves, lysophospholipids, or phosphatidic acid. The latter has been proposed to be an endogenous ionophore in neutrophils and in the endocrine islet (41, 42). However, it is possible that phosphatidic acid is further degraded by a phosphatidate-specific phospholipase A_2 to release AA (43), which could then be metabolized to lipoxygenase-dependent endogenous ionophores (55, 56).

a qualitative difference between the two groups of fatty acids in the pattern of insulin release evoked (54).

The identity of the precise lipoxygenase-derived metabolite(s) of AA mediating its actions on insulin release is not totally clarified by these studies, but our preliminary data implicate the unstable intermediates 12-hydroperoxy-ETE and perhaps LTA_4 or a similar epoxy-AA derivative. 12-HETE had no effect. The modest potency of 12-hydroperoxy-ETE to stimulate insulin release is not surprising in view of its marked lability. Furthermore, it is not clear that exogenously provided 12-hydroperoxy-ETE can reach intracellular sites where it might either act directly or be converted to active metabolites. However, by use of the glutathione-binding diethylmaleate, intracellular conversion of hydroperoxy-intermediates to both HETE and LTC_4 is blocked (20) and intracellular accumulation of hydroperoxy-ETE ensues, increasing as well the availability for conversion to LTA_4. Although direct testing of LTA_4 is not possible since it is highly unstable, use of the more stable monooxygenase-derived epoxide analogues of AA (8–10) revealed that epoxy-derivatives of AA (but not a corresponding vicinal diol) stimulate insulin release. Since recent data suggest that LTA_4 and its derivative LTB_4 act as endogenous calcium ionophores and complete secretagogues in leukocytes (55, 56), it is possible that specific lipoxygenase-dependent metabolites of AA may act similarly in the beta cell. This hypothesis is compatible with the resistance of low concentrations of the exogenous ionophore A23187 to blockade by lipoxygenase inhibitors. However, further studies will be necessary to address these interpretations conclusively.

It is likely that generation and action of lipoxygenase metabolites is interrelated with changes in calcium flux and stimulus-secretion coupling in the beta cell. It has been proposed that glucose action on the beta cell stimulates a phospholipase (57) and phospholipid turnover (58). These actions may be mediated in part by calcium-calmodulin interactions (59) since in some cells the Ca^{++}-calmodulin complex can activate phospholipase A_2 (60). Conversely, calmodulin inhibition with trifluoperazine inhibits phospholipase and generation of lipoxygenase metabolites (61) and consequent insulin release. Calcium influx may also directly activate lipoxygenase (62). The subsequent metabolism of AA released by phospholipase A_2 action (or phospholipase C with diglyceride lipase) could comprise a feed-forward mechanism for insulin release (63). In neutrophils, a similar concentration of AA as used in the present study (as well as LTB_4 and LTA_4) (55, 56, 64, 66) increases calcium gating (an effect blocked by NDGA) (64); such an action, if it also occurred in the beta cell, would promote insulin release. Thus, lipoxygenated metabolites of AA could both mediate and amplify the response to beta cell stimulation. These conclusions are consonant with the partial blockade by lipoxygenase inhibition of the effects of high-dose A23187, which appeared to activate phospholipase and thereby increase AA release.

Thus, there are close parallels between the role of lipoxygenation in the beta cell and its role as stimulus-secretion coupler in other nonendocrine secretory cells such as polymorphonuclear leukocytes, mast cells, and basophils. To take the former as an example, lipoxygenase-dependent metabolism of AA has been shown to increase hexose uptake, metabolic activity, activation of calcium influx, and granule discharge—in short, AA metabolites can act as "complete secretagogues" (55, 56, 65–67). This may be a common pathway of secretory cells in general.

These observations may be relevant to the pathogenesis of the insulin secretory defect in type II diabetics. In severe diabetes, AA synthesis may be impaired due to decreased desaturase activity (68) and its release may be impaired by diminished phospholipase activity (69) in the face of intact or heightened cyclooxygenase activity (70). Thus, a relative deficiency of AA and its stimulatory metabolites might occur. The faulty "recognition" of glucose by beta cells in human diabetes mellitus (54, 71, 72) could in part reflect an imbalance in AA release and metabolism.

Note added in proof. In preliminary studies we have observed that the unidentified peak migrating between HETE and AA in Fig. 1 is compatible with the elution characteristics of expoxy-eicosatrienoic acids (9). This suggests the possibility that a P450-dependent monooxygenase (8–10) may also be present in islet cells.

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