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# Effects of Aspirin on Nitric Oxide Formation and *De Novo* Protein Synthesis by RINm5F Cells and Rat Islets

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**SUMMARY**

Aspirin and aspirin-like drugs are the most commonly indicated agents for the treatment of inflammation. Mechanisms of action for these drugs, however, are not clearly understood. In this study, we examined the effects of aspirin on production of nitric oxide (NO), a proinflammatory mediator, and show that aspirin inhibits NO production by transformed pancreatic $\beta$ cells (RINm5F) and rat islets in a concentration-dependent manner with an $IC_{50}$ value of ~3 mM. Therapeutic concentrations of aspirin (1–5 mM) that block NO production affected neither nuclear factor-κB activation nor inducible NO synthase (iNOS) mRNA transcription but potently inhibited iNOS protein expression by both RINm5F cells and rat islets. The effects of aspirin on islet function were examined by measuring glucose-stimulated insulin secretion in the presence of various concentrations of aspirin. Aspirin (1–5 mM) did not affect insulin secretion at basal or glucose-stimulated conditions, whereas higher concentrations of aspirin (10–20 mM) significantly increased basal insulin secretion. Aspirin at high concentrations of 10 and 20 mM inhibited *de novo* protein synthesis as demonstrated by inhibition of [$^{35}$S]methionine incorporation into total islet protein and by inhibition of rabbit reticulocyte expression by Brome mosaic virus mRNA, suggesting that inhibition of iNOS expression at these high concentrations of aspirin may be due to the impairment of the translational machinery. These findings indicate that inhibition of iNOS expression and NO production may explain, in part, the beneficial effects of aspirin as an anti-inflammatory agent at therapeutic concentrations, whereas inhibition of *de novo* protein synthesis may possibly explain clinical and side effects of aspirin in the inflamed tissues and organs such as stomach and kidney that may accumulate high concentrations of aspirin.



Aspirin and ALD are the most commonly indicated agents for treatment of inflammation. These drugs have an enormous range of effects, including reducing pain or fever, dissolving corns, inhibiting blood clotting, inducing peptic ulcers, and promoting uric acid loss and fluid retention by the kidneys (1). The broad range of biological actions of aspirin have made it difficult to delineate its mechanisms of action. The most well accepted mechanism of action of aspirin is inhibition of prostaglandin biosynthesis (2). This theory, however, has been challenged because of discrepancies in clinical efficacies of aspirin in the treatment of diseases such as rheumatic fever, gout, and rheumatoid arthritis, which require much higher doses of aspirin (4–8 g/day) than required to inhibit prostaglandin production (1, 3). Moreover, salicylic acid, which is ineffective as a prostaglandin H synthase inhibitor, is nevertheless able to reduce inflammation at comparable doses to aspirin (1, 3). As alternative mechanisms of action for aspirin and ALD, the interference of cellular signaling by binding to key regulatory proteins such as G proteins (1) and inhibition of the transcriptional factor NF-κB (3) have been proposed. Nonspecific effects of aspirin and ALD due to high concentrations accumulated in some organs have also been proposed to account for the clinical and side effects of these drugs (4).

NO synthesized by iNOS has been implicated as a mediator of inflammation in rheumatic and autoimmune diseases (5–7). We (8–10) and others (11–13) have previously shown that cytokine-mediated production of NO by pancreatic $\beta$ cells plays a key role in dysfunction and destruction of $\beta$ cells associated with autoimmune diabetes. In light of the possible role of NO in the pathogenesis of autoimmune diabetes, we sought to find agents that block NO production by pancreatic $\beta$ cells. In this study, we report that aspirin blocks NO production by primary and transformed rat pancreatic $\beta$ cells

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**ABBREVIATIONS:** ALD, aspirin-like drugs; IL-1$\beta\beta$, interleukin-1$\beta$; NO, nitric oxide; iNOS, inducible nitric oxide synthase; TCA, trichloroacetic acid; NMMA, $N^G$-monomethyl-L-arginine; NF-κB, nuclear factor-κB; BMV, Brome mosaic virus; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MEM, minimum essential medium; KRB, Krebs-Ringer-bicarbonate; ECL, enhanced chemiluminescence.

at therapeutic concentrations and inhibits total *de novo* protein synthesis at higher concentrations, which may explain some of the clinical and toxic effects of aspirin.

## Materials and Methods

Male Sprague-Dawley rats (160–180 g) were purchased from Sasco (O'Fallon, MO). Collagenase type P was obtained from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Tissue culture medium (CMRL-1066), penicillin, streptomycin, Hanks' balanced salt solution, heat-inactivated fetal bovine serum, and L-glutamine were obtained from GIBCO (Grand Island, NY). The insulinoma cell line RINm5F was obtained from the Washington University Tissue Culture Support Center (St. Louis, MO). NMMA acetate was purchased from Calbiochem (San Diego, CA). Acetylsalicylic acid (solubilized in complete CMRL supplemented with 20 mM HEPES, pH 7.4), Na salicylate, and indomethacin were from Sigma Chemical (St. Louis, MO). IL-1$\beta\beta$ was from Cistron Biotechnology (Pine Brook, NJ). The cDNA probe for iNOS was a gift from Dr. Charles Rodi (Monsanto, St. Louis, MO), and the cDNA probe for cyclophilin was a gift from Dr. Jeffrey Milbrandt (Washington University). [$\alpha$-$^{32}$P]dCTP was obtained from Amersham (Arlington Heights, IL). Oligonucleotide labeling kits were from Pharmacia (Piscataway, NJ). The $^{35}$S-*trans*-labeled methionine (117 Ci/mmol) was from ICN (Costa Mesa, CA), and antisera to iNOS were obtained from Dr. Michael Marletta (University of Michigan, Ann Arbor) and Alexis Biochemicals (San Diego, CA). NF-$\kappa$B consensus oligonucleotides (5′-GATCCGAGGGGACTTTCCGCTGGGGACTTTCC-AGG-3′) and T4 polynucleotide kinase were obtained from Oncogene Science (Uniondale, NY). *In vitro* translation kits were obtained from Amersham Life Science (Buckinghamshire, UK).

**Preparation of islets.** Islets were isolated from male Sprague-Dawley rats by collagenase digestion as previously described (14). Briefly, on the day before each experiment, each pancreas was inflated with Hanks' balanced salt solution, and the tissue was isolated, minced, and digested with 6 mg of collagenase/pancreas for 9 min at 39°. Islets were separated on a Ficoll step-density gradient and then selected with a stereomicroscope to exclude any contaminating tissues. Islets (1200–1500) were then cultured overnight under an atmosphere of 95% air/5% $CO_2$ at 37° in 3 ml of complete CMRL-1066 media [CMRL-1066 containing 2 mM L-glutamine, 5.5 mM D-glucose, 10% (v/v) heat-inactivated fetal bovine serum, 100 units of penicillin/ml, and 100 $\mu$g of streptomycin/ml].

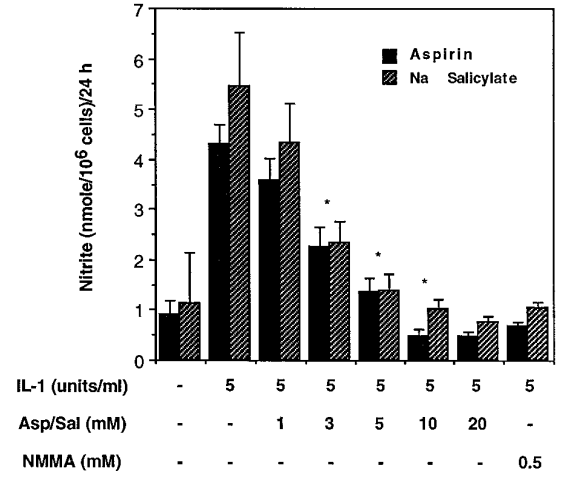


**Fig. 1.** Effects of aspirin and Na salicylate on IL-1$\beta$-induced nitrite formation by RINm5F cells. RINm5F cells ($2 \times 10^5$) were cultured at 37° for 24 hr in 200 $\mu$l of complete CMRL-1066 supplemented with 20 mM HEPES in the presence and absence of IL-1$\beta$ (10 units/ml) and indicated concentrations of aspirin, salicylic acid, and NMMA. The culture supernatant was removed, and 50-$\mu$l aliquots were mixed with 50 $\mu$l of Griess reagent. Nitrite production was determined at an absorbance of 540 nm using a Titertek Multiskan MCC/340 plate reader. Results are the average ± standard error of five individual experiments for aspirin and three individual experiments for Na salicylate containing three replicates per condition. *, Statistically significant inhibition of nitrite formation ($p < 0.01$), compared with the IL-1$\beta$-activated group, was determined by analysis of variance.

TABLE 1

**Effect of aspirin on IL-1$\beta$-induced nitrite production by rat islets**

Rat islets (200) were cultured at 37° for 24 hr in 500 $\mu$l of CMRL-1066 in the presence and absence of IL-1$\beta$ (5 units/ml) or IL-1$\beta$ plus various concentrations of 1–20 mM aspirin. As a control, 0.5 mM NMMA, a competitive inhibitor of NOS, was incubated with IL-1$\beta$. The culture medium was removed, and 50-$\mu$l aliquots were mixed with 50 $\mu$l of Griess reagent. Nitrite production was determined at an absorbance of 540 nm using a Titertek Multiskan MCC/340 plate reader. Results are the average ± standard error of six individual experiments containing three replicates in each experiment.

| Treatment | Nitrite |
|---|---|
| | *nmol/200 islets/24 hr* |
| Control | 1.3 ± 0.4 |
| IL-1$\beta$ (5 units/ml) | 4.5 ± 0.3 |
| IL-1$\beta$ + 1 (5 units/ml) | 4.5 ± 0.3 |
| IL-1$\beta$ + 1 mM aspirin | 3.6 ± 0.6 |
| IL-1$\beta$ + 3 mM aspirin | 3.1 ± 0.4 |
| IL-1$\beta$ + 5 mM aspirin | 2.6 ± 0.5 |
| IL-1$\beta$ + 10 mM aspirin | 1.2 ± 0.3 |
| IL-1$\beta$ + 20 mM aspirin | 0.8 ± 0.2 |
| IL-1$\beta$ + NMMA (0.5 mM) | 1.2 ± 0.4 |

**Nitrite determination.** RINm5F cells ($2 \times 10^5$) were cultured at 37° for 24 hr in 200 $\mu$l of complete CMRL-1066 supplemented with 20 mM HEPES, pH 7.4, in the presence and absence of IL-1$\beta$ (10 units/ml) and various concentrations of aspirin or Na salicylate as indicated in the figure legends. The culture supernatant was removed, and 50-$\mu$l aliquots were mixed with 50 $\mu$l of Griess reagent (15). Nitrite production was determined at an absorbance of 540 nm using a Titertek Multiskan MCC/340 plate reader.

**Nuclear extract preparation and electrophoretic mobility shift analysis.** Cytosolic and nuclear proteins were isolated from RINm5F cell monolayers according to the method of Flanagan *et al.* (16) Cytosolic and nuclear proteins were stored frozen at −70° before assay, and protein concentration was determined using a micro-BCA kit (Pierce, Rockford, IL). Double-stranded synthetic oligonucleotide probes for NF-$\kappa$B were end-labeled with [$\gamma$-$^{32}$P]ATP and T4 polynucleotide kinase. Cytosolic and nuclear proteins (10 $\mu$g) were incubated with oligonucleotide probes (60,000 cpm) for 35 min at room temperature, and protein/DNA complexes were resolved by polyacrylamide gel electrophoresis (17).

TABLE 2

**Effect of indomethacin on IL-1$\beta$-induced nitrite production by RINm5F cells**

RINm5F cells ($10^6$) were cultured at 37° for 24 hr in 1 ml of CMRL-1066 or CMRL-1066 containing 10 units/ml IL-1$\beta$, 100 $\mu$M indomethacin, or both IL-1$\beta$ and various concentrations of 1–100 $\mu$M indomethacin. As a control, 0.5 mM NMMA, a competitive inhibitor of NOS, was incubated with IL-1$\beta$. The culture medium was removed, and 50-$\mu$l aliquots were mixed with 50 $\mu$l of Griess reagent. Nitrite production was determined at an absorbance of 540 nm using a Titertek Multiskan MCC/340 plate reader. Results are the average ± standard error of three individual experiments containing three replicates in each experiment.

| Treatment | Nitrite |
|---|---|
| | *nmol/$10^6$ cells/24 hr* |
| Control | 0.4 ± 0.1 |
| IL-1$\beta$ (10 units/ml) | 6.3 ± 0.2 |
| IL-1$\beta$ + 1 $\mu$M indomethacin | 6.7 ± 0.2 |
| IL-1$\beta$ + 10 $\mu$M indomethacin | 6.7 ± 0.1 |
| IL-1$\beta$ + 100 $\mu$M indomethacin | 7.0 ± 0.2 |
| 100 $\mu$M indomethacin | 0.7 ± 0.1 |
| IL-1$\beta$ + NMMA (0.5 mM) | 0.3 ± 0.1 |

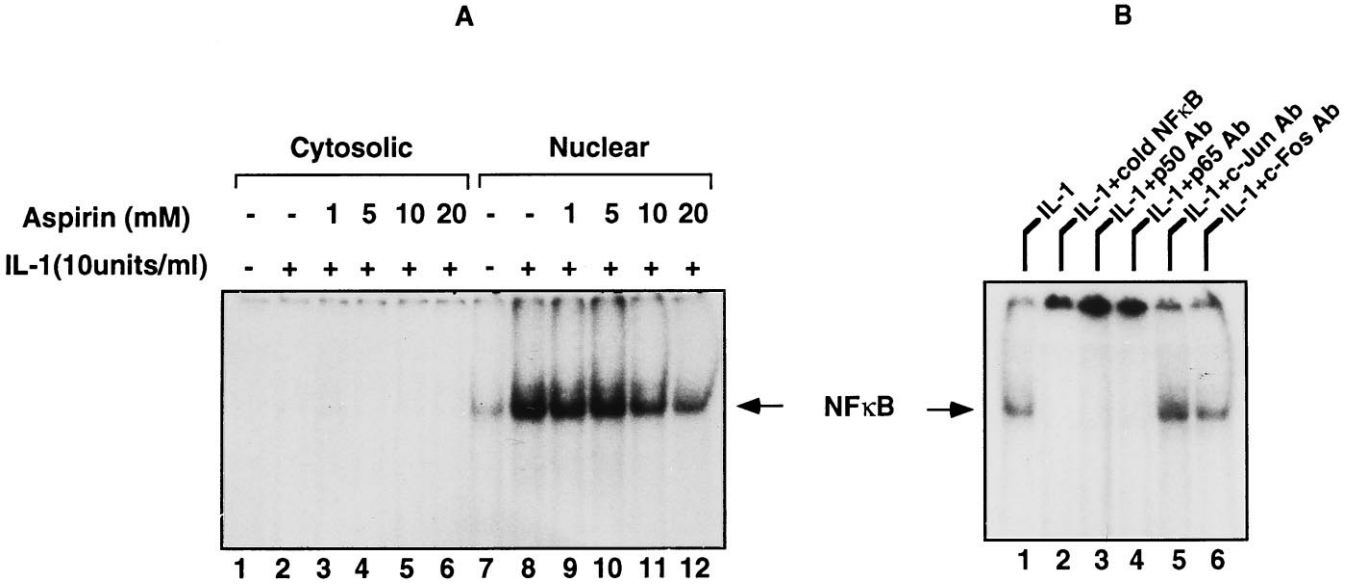


**Fig. 2.** Effect of aspirin on IL-1$\beta$-induced translocation of NF-$\kappa$B from cytosol to the nucleus. A, RINm5F cells ($5 \times 10^7$) were treated with vehicle (*lanes 1* and *7*), 10 units/ml IL-1$\beta$ (*lanes 2* and *8*), and IL-1$\beta$ plus indicated concentrations of aspirin (*lanes 3–6* and *9–12*) for 1 hr. Cytosolic and nuclear proteins were prepared from these cells according to the method of Flanagan *et al.* (16) and stored frozen at −70° before assay. Gelshift assays were performed as described in Materials and Methods. B, Nuclear proteins isolated from cells treated with IL-1$\beta$ (10 units/ml) were incubated alone (*lane 1*) or with 150-fold excess amounts of unlabeled oligo probes (*lane 2*), rabbit antiserum (0.2 $\mu$g/condition) of NF-$\kappa$B p50 (*lane 3*) or p65 (*lane 4*), c-*Jun* (*lane 5*), or c-*Fos* (*lane 6*) for 35 min, followed by gel electrophoresis. Results are representative of three individual experiments.

**RNA isolation and Northern blot analysis.** RINm5F cells ($5–7 \times 10^7$) or islets (1500) were treated with IL-1$\beta$ for the indicated time periods as shown in the figure legends, followed by washing three times with PBS, pH 7.4, and then solubilization in 3.5 ml of 4 M guanidinium isothiocyanate. Total RNA from lysates was sedimented by ultracentrifugation on a cushion of 5.7 M cesium chloride (18). Total cellular RNA (20–50 $\mu$g) was denatured and fractionated electrophoretically using a 1.2% agarose gel containing 3% formaldehyde and transferred by blotting to nylon membranes. Blots were prehybridized overnight at 42° with prehybridization buffer (19). Hybridization was carried out overnight at 42° in fresh prehybridization buffer containing $^{32}$P-labeled cDNA probes. cDNA probes were labeled with [$\alpha$-$^{32}$P]dCTP using a nick-translation kit according to the supplier's (Pharmacia) instructions. After hybridization, the membranes were extensively washed with buffer [0.1% SDS and 0.1× standard saline citrate (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0)] at 42° and autoradiographed with intensifying screens at −70°.

**Determination of iNOS protein expression.** RINm5F cells ($5 \times 10^5$ in 500 $\mu$l complete CMRL-1066 supplemented with 20 mM HEPES) were activated with 10 units/ml IL-1$\beta$ and various concentrations of aspirin (1–20 mM) for 24 hr. Cells were washed three times with PBS, pH 7.4, and solubilized in Laemmli's sample buffer (30 $\mu$l). Samples were denatured, run on a 10% SDS acrylamide gel, and transferred to Hybond ECL Nitrocellulose (Amersham) and immunoblot analysis was performed using rabbit iNOS antiserum (1:2000) and peroxidase-conjugated donkey anti-rabbit IgG (1:7000) as the primary and the secondary antisera, respectively. Proteins were detected by enhanced chemiluminescence (ECL; Amersham). iNOS protein expression by rat islets was determined by immunoprecipitation of iNOS from metabolically labeled rat pancreatic islets as previously described (20). Briefly, islets (200 in 500 $\mu$l of complete CMRL-1066) were washed three times with 500 $\mu$l of methionine-deficient MEM (9 parts MEM without methionine/1 part MEM containing methionine) supplemented with 20 mM HEPES (pH 7.4) and incubated at 37° for 5 hr. Islets were then treated with 5 units/ml IL-1$\beta$, various concentrations of aspirin (1–20 mM), and 215 $\mu$Ci of [$^{35}$S]methionine *Trans*-Label (ICN) and further incubated for 19 hr. Islets were then isolated by centrifugation (20 sec at 14,000 rpm), washed, and processed for immunoprecipitation of iNOS using a rabbit affinity-purified polyclonal antibody raised against a peptide corresponding to mouse macrophage amino acid residues 1131–1144 according to the modified method of Corbett *et al.* (21).

**Glucose-stimulated insulin secretion.** Isolated islets (150/1 ml of complete CMRL-1066 supplemented with 20 mM HEPES, pH 7.4, to avoid changes in pH due to high concentrations of aspirin) were exposed to various concentrations of aspirin (1–20 mM) for 24 hr at 37°. After the exposure, islets were washed three times (1 ml/wash) in KRB buffer (containing 25 mM HEPES, 115 mM NaCl, 24 mM $NaHCO_3$, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, pH 7.4) containing 3 mM D-glucose and 0.1% bovine serum albumin. Groups of 20 islets were counted into 10 × 75-mm siliconized borosilicate tubes and preincubated for 30 min in 200 $\mu$l of the same buffer under an atmosphere of 95% air/5% $CO_2$ at 37° with shaking. The preincubation buffer was removed, and glucose-stimulated insulin secretion was initiated by the addition of 200 $\mu$l of fresh KRB containing either 3 or 20 mM D-glucose followed by a 30-min incubation. Insulin secretion was determined in the incubation buffer by insulin radioimmunoassay.

**[$^{35}$S]Methionine incorporation into rat islets.** Isolated rat islets (100) were cultured at 37° for 24 hr in 1 ml of complete CMRL-1066 supplemented with 20 mM HEPES in the presence of 10 $\mu$M cycloheximide or the indicated concentrations of aspirin. [$^{35}$S]Methionine (14.3 $\mu$Ci/$\mu$l) was included in all samples. After the 24-hr culture period, the islets were distributed (20 islets/tube) into 1.5-ml polyallomer tubes. The islets were washed three times with fresh CMRL-1066 medium to remove unincorporated radiolabel. Then, 500 $\mu$l of ice-cold TCA (10% w/v) was added to precipitate islet protein. The islets were sequentially washed and pelleted three times with the ice-cold TCA solution. The $^{35}$S content of the pellet was then determined by liquid scintillation counting (model 1500; Packard Instruments, Downers Grove, IL).

*In vitro* translation of BMV mRNA. Translational reactions (final, 50 $\mu$l), including biotin-Lys-tRNA (1 $\mu$l) and rabbit reticulocyte lysate (20 $\mu$l), were prepared following the manufacturer's instructions (Amersham Life Science). Increasing concentrations of aspirin (1–20

mM) were added to the reaction mixtures and incubated at 30° for 1 hr, and the reaction was terminated by placing the samples on ice. Samples were denatured, run on a 10% SDS acrylamide gel, and transferred to Hybond ECL Nitrocellulose. Blots were blocked for 1 hr at room temperature in 5% blocking agent (included in the kit) in PBS-T (80 mM $Na_2HPO_4$, 20 mM $NaH_2PO_4$, 100 mM NaCl, 0.1% Tween 20), followed by incubation in 10 ml of PBS-T containing the streptavidin-horseradish peroxidase conjugate (1:1000 dilution). Four different proteins encoded by BMV virus mRNA were detected by ECL.

**Statistics.** Statistical comparisons were made between groups using a one-way analysis of variance. Significant differences ($p < 0.05$) were evaluated using Scheffé F test *post hoc* analysis.

## Results

Aspirin inhibited IL-1β-induced production of nitrite, an oxidized form of NO, by the insulinoma cell line RINm5F in a concentration-dependent manner with an $IC_{50}$ value of ~3 mM (Fig. 1, *black bars*). The NOS inhibitor NMMA (0.5 mM) also inhibited IL-1β-induced nitrite production to control levels, and aspirin in the absence of IL-1β had no effect on nitrite levels (data not shown). The same dose-dependent effects of aspirin on IL-1β-induced production of nitrite were observed with isolated rat islets (Table 1). The acetyl moiety of aspirin (acetylsalicylate) does not seem to be required for the inhibition of nitrite generation because Na salicylate exhibits a similar concentration-dependent inhibition of nitrite production by RINm5F cells compared with aspirin (Fig. 1, *hatched bars*). Indomethacin (1–100 μM), another nonsteroidal anti-inflammatory drug, which is structurally dissimilar to aspirin, had no effect on IL-1β-induced nitrite formation (Table 2), suggesting that the ability of aspirin to block IL-1β-induced nitrite formation may be unique among nonsteroidal anti-inflammatory drugs.

Because NF-κB is a primary transcriptional factor in the regulation of iNOS (22, 23), we initially examined the effects of aspirin at therapeutic concentrations (1–5 mM) on IL-1β-induced NF-κB activation. Aspirin (1–5 mM) did not inhibit IL-1β-induced translocation of NF-κB to the nucleus of RINm5F cells (Fig. 2A, *lanes 9 and 10*), whereas high concentrations of aspirin (10–20 mM) significantly blocked NF-κB translocation (Fig. 2A, *lanes 11 and 12*). Cytosolic proteins isolated in parallel with the nuclear proteins did not form protein/DNA complexes (Fig. 2A, *lanes 1–6*). The addition of an excess amount (150-fold) of unlabeled NF-κB oligonucleotides to the reaction mixture prevented the appearance of the NF-κB band (Fig. 2B, *lane 2*). Supershift assays using antibodies for NF-κB subunits p50 and p65 resulted in the complexes at the top of the gels being retained (Fig. 2B, *lanes 3 and 4*). Antibodies for c-*Jun* and c-*Fos*, however, did not affect the migration of the NF-κB band (Fig. 2B, *lanes 5 and 6*). These results suggest that the site of action of aspirin (1–5 mM) to block NO formation is not at the level of NF-κB activation.

To determine whether aspirin inhibits NO production at the level of transcription, we next examined the effects of aspirin on IL-1β-induced iNOS mRNA transcription by RINm5F cells and rat islets using Northern blot analysis. Aspirin (1–10 mM) did not inhibit IL-1β-induced iNOS mRNA expression by both RINm5F cells and rat islets (Fig. 3, *top* and *bottom*). However, 20 mM aspirin completely blocked iNOS mRNA expression by RINm5F cells (Fig. 3, *top*, *first blot*, *lane 6*). Complete inhibition of iNOS mRNA transcription in the presence of 20 mM aspirin may be due to nonspecific or cytotoxic effects of aspirin as reflected by increased basal insulin secretion (see Fig. 7). Transcriptional machinery, however, seems to be intact in the presence of 20 mM aspirin because cyclophilin mRNA is minimally affected (Fig. 3, *top*, *second blot*, *lane 6*). These experiments suggest that aspirin (1–10 mM) does not affect iNOS transcription by RINm5F cells and rat islets determined after a 6-hr exposure to IL-1β and aspirin.

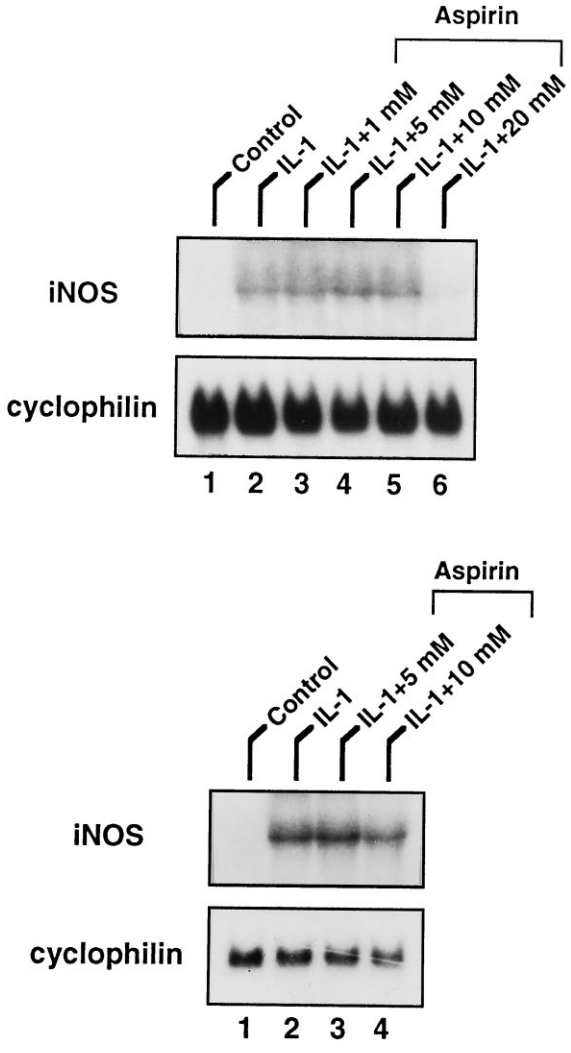


**Fig. 3.** Effect of aspirin on IL-1β-induced iNOS mRNA expression by RINm5F cells and rat islets. RINm5F cells ($5–8 \times 10^7$) (*top*) or rat islets (1500) (*bottom*) were exposed to IL-1β and/or various concentrations of aspirin for 6 hr as indicated. Northern blot analysis was performed by the method described in Materials and Methods. Results are representative of three individual experiments.

To examine whether aspirin down-regulates iNOS mRNA expression by decreasing its half-life, which might be detectable at a later time point, we examined the effects of aspirin on the time course of iNOS mRNA expression by RINm5F cells. We have previously demonstrated that IL-1β-induced iNOS mRNA expression by RINm5F cells is detectable after 3 hr, peaks at ~6 hr, and decreases significantly after a 9-hr exposure to IL-1β (24). Therefore, we examined the 0-, 6-, and 9-hr time points. Aspirin (5 and 10 mM) did not affect iNOS mRNA expression after a 6-hr exposure (Fig. 4A, *lanes 3–5*),

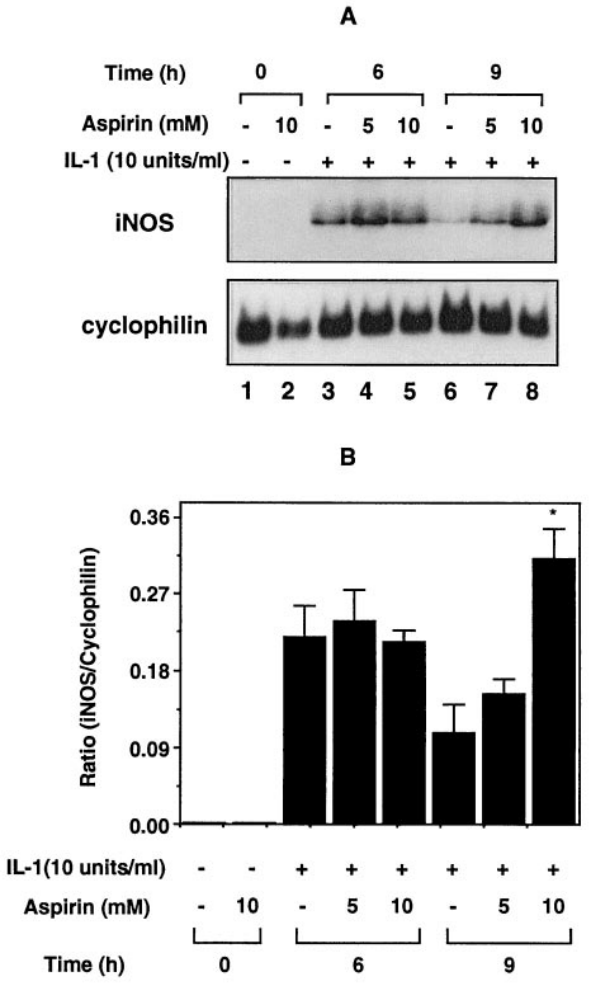


**Fig. 4.** Effect of aspirin on time course of IL-1$\beta$-induced iNOS mRNA expression by RINm5F cells. A, RINm5F cells (5–8 $\times$ $10^7$) were treated with IL-1$\beta$ (10 units/ml) $\pm$ 5 or 10 mM aspirin for the indicated time periods. Northern blot analysis was performed by the method described in Materials and Methods. Results are representative of three individual experiments. B, Autoradiograms were scanned by laser densitometry (Molecular Dynamics). Intensity of iNOS and cyclophilin bands was quantified by integrating the area under the curve (Molecular Dynamics Image Quant software), and the ratio of iNOS/cyclophilin was calculated. Results are the average $\pm$ standard error of three individual experiments. *, Statistically significant increase in iNOS mRNA accumulation by the group treated with IL-1$\beta$ and 10 mM aspirin for 9 hr ($p <$ 0.01), compared with the control group treated with IL-1$\beta$ for 9 hr (*sixth bar*), was determined by analysis of variance.

confirming our results shown in Fig. 3. However, aspirin increased the levels of iNOS mRNA in a concentration-dependent manner (Fig. 4A, compare *lane 6* with *lanes 7 and 8*) determined after a 9-hr exposure to IL-1$\beta$ and aspirin, suggesting that aspirin increases the steady state levels of iNOS mRNA. Fig. 4B shows quantification of iNOS mRNA expression by laser densitometry, normalized by calculating the ratio of iNOS over cyclophilin. iNOS mRNA expression by the group treated with IL-1$\beta$ and 10 mM aspirin for 9 hr (*eighth bar*) is significantly increased compared with the control group (*sixth bar*).

Because aspirin (1–5 mM) did not affect iNOS mRNA transcription, the effects of aspirin on iNOS protein expression by RINm5F cells and rat islets were examined by Western blot analysis and immunoprecipitation, respectively. As shown in Fig. 5, A and B, aspirin inhibits IL-1$\beta$-induced iNOS protein expression in a concentration-dependent manner by both RINm5F cells and rat islets, similar to the inhibitory effects of aspirin on nitrite formation. Significant inhibition of iNOS expression is observed at 3 and 5 mM aspirin (Fig. 5, A and B, *lanes 4 and 5*), and complete inhibition is observed at 20 mM aspirin (Fig. 5, A and B, *lane 7*) after a 24-hr exposure to IL-1$\beta$. Fig. 6, A and B, shows quantification of iNOS protein expression by the two cell types by laser densitometry. These results suggest that aspirin (1–5 mM) blocks NO formation at the level of protein synthesis.

To determine whether the inhibitory effects of aspirin on iNOS expression are due to the cytotoxic effects of aspirin caused by its high concentration, we examined the effects of aspirin on islet function by measuring glucose-stimulated insulin secretion from isolated rat islets. Because IL-1$\beta$ by itself inhibits glucose-stimulated insulin secretion by rat islets, we did not include IL-1$\beta$ for these experiments. Aspirin (1–5 mM) did not affect insulin secretion at basal or glucose-stimulated conditions, whereas higher concentrations of as-

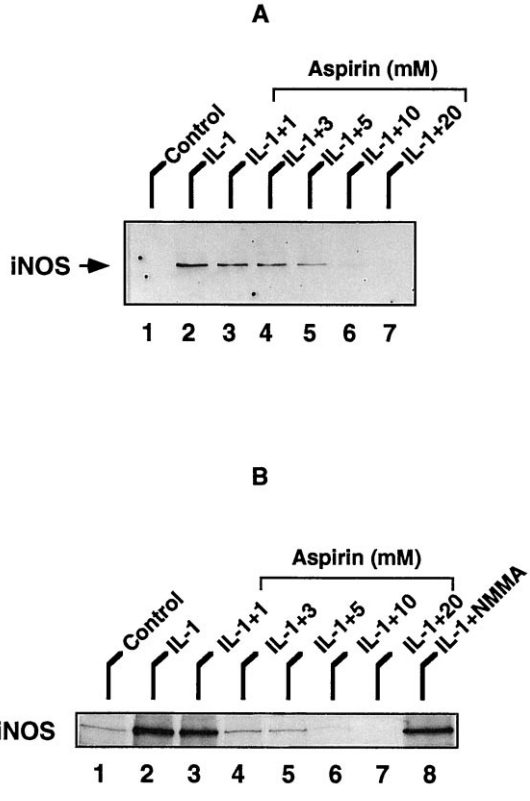


**Fig. 5.** Effect of aspirin on IL-1$\beta$-induced iNOS expression by RINm5F cells and rat islets. A. RINm5F cells (5 $\times$ $10^5$ in 500 $\mu$l complete CMRL-1066 supplemented with 20 mM Hepes) were activated with 10 units/ml IL-1$\beta$ and various concentrations of aspirin (1–20 mM) for 24 hr. Cells were washed three times with PBS, pH 7.4, and solubilized in Laemmli's sample buffer (30 $\mu$l). Samples were processed for Western blot analysis as described in Materials and Methods. B, Islets (200 in 500 $\mu$l complete CMRL-1066) were washed three times with methionine-deficient MEM supplemented with 20 mM HEPES and incubated at 37° for 5 hr. Islets were then treated with 5 units/ml IL-1$\beta$, various concentrations of aspirin (1–20 mM), and 215 $\mu$Ci [$^{35}$S]methionine *Trans*-Label (ICN) and further incubated for 21 hr. Islets were then isolated by centrifugation, washed, and processed for immunoprecipitation of iNOS using a rabbit affinity-purified polyclonal antibody as described in Materials and Methods. Results are representative of three individual experiments.

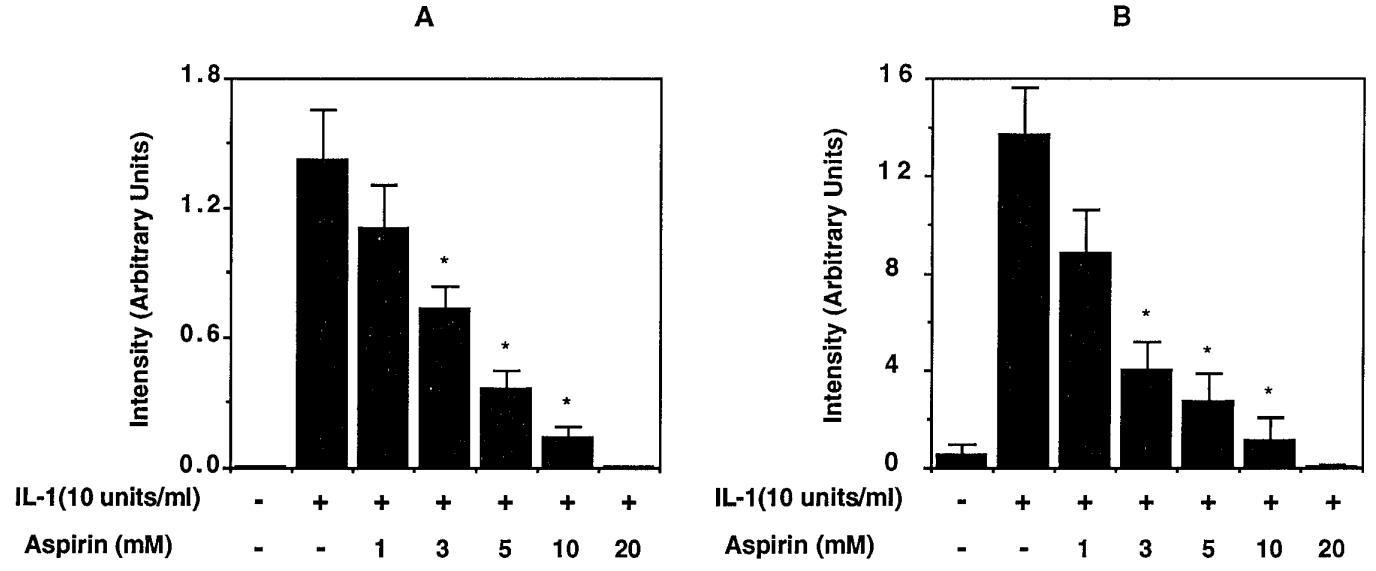


**Fig. 6.** Effects of aspirin on IL-1$\beta$-induced iNOS expression by RINm5F cells and rat islets. A and B, Western blot or Immunoprecipitation of iNOS was carried out as described in the legend to Fig. 5. Autoradiograms were scanned by laser densitometry. Intensity of iNOS bands was quantified by integrating the area under the curve. Results are the average ± standard error of three individual experiments. *, Statistically significant inhibition of iNOS protein expression ($p < 0.01$), as compared with IL-1$\beta$ treated group, was determined by analysis of variance.

pirin (10–20 mM) significantly increased basal insulin secretion (Fig. 7). These results indicate that aspirin at high concentrations has deleterious effects on islet function. Thus, the inhibition of NO formation at these high concentrations (10–20 mM) may be due to cytotoxic effects.

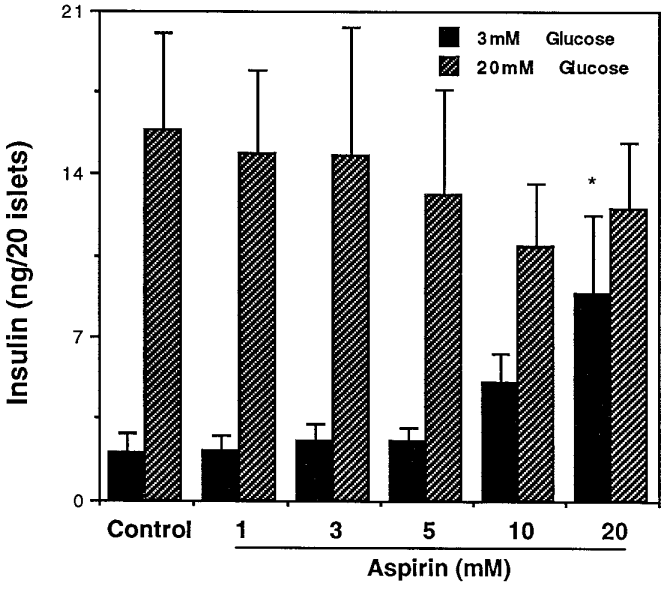


**Fig. 7.** Effects of aspirin on basal and glucose-stimulated insulin secretion by rat islets. Isolated islets (150 in 1 ml of complete CMRL-1066 supplemented with 20 mM HEPES) were exposed to various concentrations of aspirin (1–20 mM) for 24 hr at 37°. After the exposure, islets were washed three times (1 ml/wash) in KRB buffer containing 3 mM D-glucose and 0.1% bovine serum albumin. Groups of 20 islets were counted into 10 × 75-mm siliconized borosilicate tubes and preincubated for 30 min in 200 $\mu$l of the same buffer under a atmosphere of 95% air/5% $CO_2$ at 37° with shaking. The preincubation buffer was removed, and glucose-stimulated insulin secretion was initiated by the addition of 200 $\mu$l of fresh KRB containing either 3 or 20 mM D-glucose followed by a 30-min incubation. Insulin secretion was determined in the incubation buffer by insulin radioimmunoassay. Results are the average ± standard error of three individual experiments containing three replicates per condition. *, Statistically significant insulin secretion ($p < 0.01$), compared with the untreated control, was determined by analysis of variance.

Because high concentrations of aspirin had deleterious effects on islet function, we examined the effects of aspirin on cell viability by trypan blue dye exclusion and total protein synthesis by [$^{35}$S]methionine incorporation into rat islets. Incubation of RINm5F cells for 24 hr in the presence of 10 and 20 mM aspirin did not affect cell viability based on trypan blue dye exclusion experiments (data not shown). These concentrations of aspirin, however, significantly inhibited total *de novo* protein synthesis to a level in the case of 20 mM aspirin comparable to cycloheximide (10 $\mu$M), as shown in Fig. 8. Therapeutic concentrations of aspirin (1–5 mM) that inhibited iNOS protein expression did not block total protein synthesis. Although 5 mM aspirin decreased protein synthesis, this effect was not statistically significant. Similar results were obtained with RINm5F cells and RAW 264.7 cells (data not shown).

Next, we examined whether the inhibition of total *de novo* protein synthesis by aspirin at the concentrations of 10 and 20 mM is due to the impairment of the translational machinery by studying the effects of aspirin on *in vitro* translation of BMV mRNA. As shown in Fig. 9, aspirin (10–20 mM) inhibited the translation of four BMV viral mRNAs coding for proteins with molecular masses of 109, 94, 35, and 20 kDa using a rabbit reticulocyte lysate system. Aspirin (1–5 mM) did not inhibit the translation of the viral mRNAs, supporting the results shown in Fig. 8. The two higher molecular mass proteins, 109 and 94 kDa, are reported to often run as one band (manufacturer's instruction manual), as indicated in Fig. 9 (*110/97*). Exclusion of the BMV mRNA or incubation with cycloheximide (10 $\mu$M) completely prevents protein synthesis (Fig. 9, *lanes 1 and 3*, respectively). These experiments suggest that aspirin at 10 and 20 mM concentrations impair the translational machinery. Therefore, the inhibition of iNOS expression under these high concentrations may be, in part, due to its impairment of the translational machinery in RINm5F cells and rat islets.

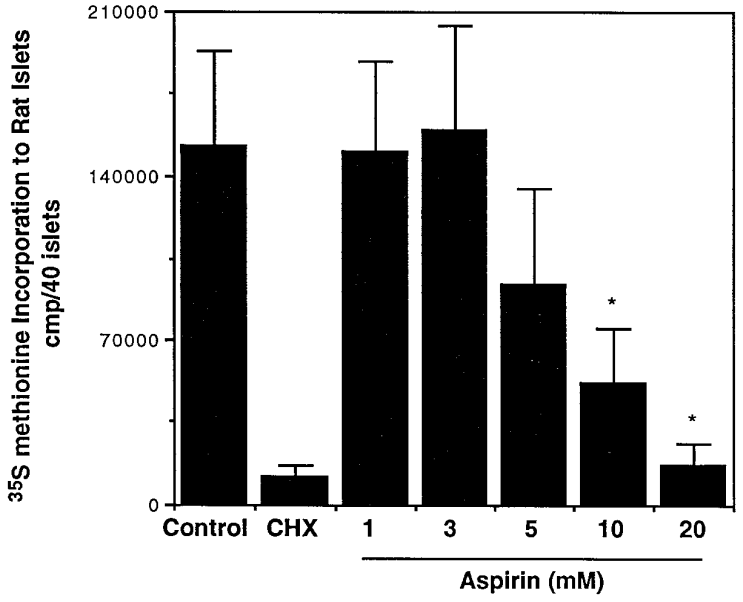


**Fig. 8.** Effect of aspirin on [$^{35}$S]methionine incorporation into rat islets. Isolated rat islets (100) were cultured at 37° for 24 hr in 1 ml of complete CMRL-1066 supplemented with 20 mM HEPES in the presence of 10 $\mu$M cycloheximide, or the indicated concentrations of aspirin. [$^{35}$S]methionine (14.3 $\mu$Ci/$\mu$l) was included in all samples. After the 24-hr culture period, the islets were distributed (20 islets/tube) into 1.5-ml polyallomer tubes. The islets were washed three times with fresh CMRL-1066 medium to remove unincorporated radiolabel. Then, 500 $\mu$l ice-cold TCA (10% w/v) was added to precipitate islet protein. The islets were sequentially washed and pelleted three times with the ice-cold TCA solution. The $^{35}$S content of the pellet was then determined by liquid scintillation counting. Results are the average ± standard error of three individual experiments containing three replicates per condition. Statistically significant inhibition of [$^{35}$S]methionine incorporation ($p < 0.01$), compared with the untreated control, was determined by analysis of variance.

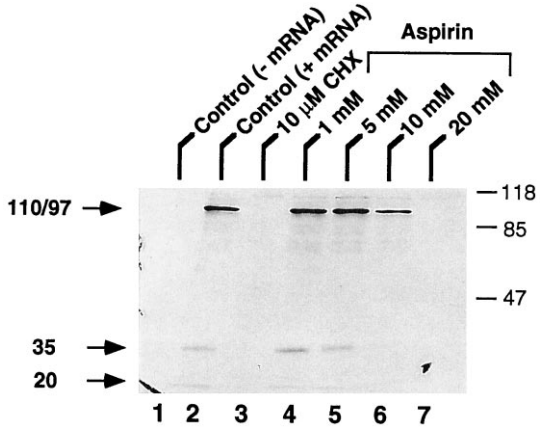


**Fig. 9.** Effect of aspirin on *in vitro* translation of BMV mRNA. Aspirin (1–20 mM) was added to translational reactions (final, 50 $\mu$l), including biotin-Lys-tRNA (1 $\mu$l) and rabbit reticulocyte lysate (20 $\mu$l), and incubated at 30° for 1 hr. The reaction was terminated by placing samples on ice. Samples were then denatured, run on a 10% SDS acrylamide gel, and transferred to Hybond ECL Nitrocellulose. Blots were blocked for 1 hr at room temperature in 5% blocking agent in PBS-T, followed by incubation in 10 ml of PBS-T containing the streptavidin-horseradish perioxidase conjugate (1:1000 dilution). Four different proteins encoded by BMV virus mRNA were detected by ECL. Results are representative of three individual experiments.

## Discussion

In this study, we report that aspirin at therapeutic concentrations (1–5 mM) inhibits NO production at the level of iNOS protein expression by RINm5F cells and rat islets. Higher concentrations of aspirin (10–20 mM) stimulated basal insulin secretion and inhibited NF-$\kappa$B activation, iNOS mRNA transcription, and total *de novo* protein synthesis. Inhibition of NO production by islets at therapeutic concentrations of aspirin suggests that this may explain the beneficial effects of aspirin as an anti-inflammatory agent.

Although total *de novo* protein synthesis is greatly reduced at high concentrations of aspirin (10–20 mM), cells seem to be intact functionally based on several observations: (i) IL-1$\beta$-induced NF-$\kappa$B translocation to the nucleus still occurs, although at a diminished rate; (ii) cyclophilin mRNA expression is minimally affected; and (iii) trypan blue exclusion experiments also suggest that 20 mM aspirin does not affect viability of these cells (data not shown). Increases in basal insulin secretion, however, suggest some cytotoxic effects of aspirin at these high concentrations on cellular function.

The lack of an effect of aspirin on IL-1$\beta$-induced iNOS mRNA expression except at a high concentration of 20 mM (Fig. 4) is intriguing. Although 10 mM aspirin significantly blocks (>50%) translocation of NF-$\kappa$B to the nucleus, this inhibition is not reflected by reduced iNOS mRNA expression, which is expected because NF-$\kappa$B is the primary transcriptional factor in the regulation of iNOS mRNA expression (22, 23). It seems that the level of NF-$\kappa$B translocated to the nucleus in the presence of 10 mM aspirin is still sufficient to fully activate the transcription of iNOS, suggesting that there may exist a threshold level of NF-$\kappa$B required for iNOS gene transcription.

The site of action of aspirin or Na salicylate in the signaling pathway of NO production has been reported to vary among different cell types. Farivar *et al.* (25) reported that Na salicylate (4 mM) diminishes steady state levels of iNOS mRNA in neonatal cardiac fibroblasts. On the other hand, Kepka-Lenhart *et al.* (26) reported that aspirin (3–10 mM) inhibits cytokine-induced NO production and expression of iNOS protein without inhibiting induction of iNOS mRNA in the murine macrophage cell line, RAW 264.7. Amin *et al.* (27) also reported that aspirin ($IC_{50}$ = 3 mM), but not Na salicylate, inhibits NO production at the level of iNOS protein expression and also inhibits enzymatic activity of iNOS in RAW 264.7 cells. In rat islets and insulinoma RINm5F cells, we report that aspirin (1–5 mM) blocks NO production at the level of iNOS protein expression without affecting iNOS mRNA levels. Aspirin at higher concentration (5–10 mM) increases the steady state levels of iNOS mRNA measured after a 9-hr exposure to IL-1$\beta$ and aspirin (Fig. 4), probably reflecting the accumulation of iNOS mRNA due to the inhibition of iNOS protein expression at the translational level.

Specific mechanisms by which aspirin inhibits iNOS protein expression by RINm5F cells and rat islets are not clear.

High concentrations of aspirin (10–20 mM) seem to inhibit iNOS expression, in part, by impairing the translational machinery based on *in vitro* translation of BMV mRNA (Fig. 9). However, the mechanisms involved in the inhibition of iNOS expression at lower concentrations of aspirin are currently unknown. Although unlikely, we could not rule out the possibility that iNOS degradation may be accelerated by aspirin.

The results of our current study suggest that relatively high concentrations of aspirin (10–20 mM) block NF-κB activation (Fig. 2) and *de novo* protein synthesis (Figs. 8 and 9). The acidic property of aspirin ($pK_a \sim 4$) facilitates its cellular uptake in acidic environments due to increased lipophilicity (4). Therefore, organs that contain acidic compartments, such as stomach, kidney, and inflamed tissues, may attain several-fold higher concentrations of aspirin compared with plasma levels. Thus, *in vitro* studies elucidating various mechanisms of action for aspirin at high concentrations, including inhibition of NF-κB activation (3), cellular kinases (28), and *de novo* protein synthesis (current study), may be applicable to *in vivo* situations.

In summary, our results indicate that aspirin at therapeutic concentrations of 1–5 mM significantly inhibits IL-1β-induced NO production from both primary and transformed β cells. The primary mechanism responsible for this effect is inhibition of iNOS protein expression that may be mediated at a post-transcriptional level by aspirin because neither NF-κB activation nor iNOS mRNA levels were significantly altered. These findings may explain, in part, the beneficial effects of aspirin and aspirin-like drugs when used as anti-inflammatory agents at these therapeutic concentrations.

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