Recent evidence supports a regulatory role for the calcium-independent phospholipase A₂ (iPLA₂) in the antiviral response of inducible nitric-oxide synthase (iNOS) expression by macrophages. Because two mammalian isoforms of iPLA₂ (iPLA₂β and iPLA₂γ) have been cloned and characterized, the aim of this study was to identify the specific isoform(s) in macrophages that regulates the expression of iNOS in response to virus infection. Bromoenol lactone (BEL), a suicide substrate inhibitor of iPLA₂, inhibits the activity of both isoforms at low micromolar concentrations. However, the R- and S-enantiomers of BEL display ~10-fold greater potency for inhibition of the enzymatic activity of iPLA₂γ and iPLA₂β, respectively. In this study, we show that the iPLA₂β-selective (S)-BEL inhibits encephalomyocarditis virus (EMCV)-induced iNOS expression, nitric oxide production, and iPLA₂ enzymatic activity in macrophages in a concentration-related manner that closely resembles the inhibitory properties of racemic BEL. cAMP response element-binding protein (CREB) is one downstream target of iPLA₂ that is required for the transcriptional activation of iNOS in response to virus infection, and consistent with the effects of BEL enantiomers on iNOS expression, (S)-BEL more effectively inhibits EMCV-induced CREB phosphorylation than (R)-BEL in macrophages. Using macrophages isolated from iPLA₂β-null mice, virus infection fails to stimulate iNOS mRNA accumulation and protein expression, thus providing genetic evidence that iPLA₂β is required for EMCV-induced iNOS expression. These findings provide evidence for a signaling role for iPLA₂β in virus-induced iNOS expression by macrophages.

The dsRNA³-dependent protein kinase (PKR) is a primary mediator of antiviral activities within infected cells. PKR, a 65–68-kDa serine-threonine protein kinase, is activated by binding to dsRNA that accumulates during viral infection and coordinates the antiviral response by inhibiting protein translation, up-regulating antiviral and proinflammatory gene expression, and inducing apoptosis (1, 2). PKR suppresses protein translation in virus-infected cells by phosphorylation and subsequent inhibition of the eukaryotic translation initiation factor eIF2α, whereas PKR-mediated gene transcription appears to be due to activation of transcription factors such as nuclear factor (NF)-κB (3).

Whereas the regulation of the antiviral response has classically been thought to be mediated by PKR, recent reports support the existence of additional PKR-independent pathways that participate in antiviral activities within infected cells. In particular, treatment of PKR⁻/⁻ mouse embryonic fibroblasts with dsRNA or infection with encephalomyocarditis virus (EMCV) stimulates inhibitory protein κB (IκBα) degradation as well as NF-κB nuclear localization, DNA binding activity, and promoter activation, suggesting that PKR is not absolutely required for virus-induced activation of NF-κB-mediated gene expression (4). Furthermore, experimental evidence suggests PKR-independent pathways regulate dsRNA- and virus-induced expression of the NF-κB-responsive proinflammatory genes interleukin-1 (IL-1) (5), cyclooxygenase-2 (6), and the inducible isoform of nitric-oxide synthase (iNOS) (7) by macrophages.

The expression of iNOS and subsequent production of nitric oxide appear to play a primary role in innate immunity because viral infection of rodent (8) and human (9) macrophages stimulates iNOS expression and iNOS-deficient mice have increased viral titers and higher mortality than wild-type mice following virus infection (10, 11). In an attempt to elucidate the mechanisms that regulate proinflammatory gene expression in macrophages during viral infection, we have recently identified a role for calcium-independent phospholipase A₂ (iPLA₂) in a novel PKR-independent signaling pathway that participates in the transcriptional regulation of iNOS (7). Treatment of macrophages with dsRNA or infection with EMCV results in an increase in membrane-associated iPLA₂ enzymatic activity and iNOS mRNA accumulation and protein expression. The suicide carditis virus; ERK, extracellular signal-regulated kinase; GADPH, gluteraldehyde 3-phosphate dehydrogenase; IFN, interferon; IL-1, interleukin 1; iNOS, inducible isoform of nitric-oxide synthase; iPLA₂, calcium-independent phospholipase A₂; NF, nuclear factor; puf, plaque-forming unit; PKA, protein kinase A; PKR, dsRNA-dependent protein kinase; poly(IC), polyinosinic-polycytidylic acid; STAT, signal transducer and activator of transcription; RT, reverse transcription.
substrate inhibitor of iPLA₂, bromoeno lactone (BEL), inhibits EMCV-induced iPLA₂ activation and iNOS expression in a concentration-dependent manner, supporting a role for iPLA₂ in the regulation of iNOS expression by macrophages. The mechanism by which iPLA₂ mediates the transcriptional activation of iNOS appears to be associated with membrane phospholipid hydrolysis and liberation of a lipid-derived mediator that is capable of activating downstream signaling targets that participate in the expression of iNOS. Importantly, we have shown that the inhibitory effects of BEL on dsRNA-induced iNOS expression by RAW264.7 cells can be overcome by the exogenous addition of a lipid product resembling those generated by iPLA₂ activity, lysoplasmalogenol (7). iPLA₂ appears to selectively regulate iNOS expression in response to viral infection because inhibition of iPLA₂ activity does not attenuate virus-induced expression of other proinflammatory genes such as IL-1 or cyclooxygenase-2 (6, 7).

iPLA₂ catalyzes the hydrolysis of the sn-2 fatty acid sub- stituent of membrane phospholipids to generate a free fatty acid and a lysophospholipid (12, 13). Two mammalian isozymes of iPLA₂ (iPLA₂β and iPLA₂γ) have been identified and characterized (14, 15), and their enzymatic activity has been implicated in diverse cellular functions including homeo- static membrane phospholipid metabolism (16), secretogogue-induced insulin secretion from pancreatic β-cells (17), cell proliferation (18), and apoptosis (19). Historically, the functions of iPLA₂ have been delineated and characterized primarily by sensitivity to the suicide substrate inhibitor BEL. Although BEL is 1000-fold more selective for iPLA₂ than other phospholipases (20, 21), distinguishing the biological roles of iPLA₂β and iPLA₂γ by pharmacologic means had not been possible until the recent recognition that these two enzymes are differentially susceptible to inhibition by the enantiomers of BEL (R)-BEL and (S)-BEL. Studies by Gross and co-workers (22) have shown that (R)-BEL is 10-fold more selective for iPLA₂γ, whereas (S)-BEL is 10-fold more selective for iPLA₂β. However, it is important to note that Bel enantiomers do not display absolute specificity for iPLA₂ isozymes because a significantly high enough concentration of either (R)- or (S)-BEL will inhibit both iPLA₂β and iPLA₂γ. Accordingly, when used at submaximally effective concentrations, the differential sensitivity of iPLA₂ to inhibition by the enantiomers of BEL allows for the identification of the iPLA₂ isoform that mediates a given biological response.

Using this pharmacological approach, the identity of the iPLA₂ isoform(s) that mediates the antiviral response of iNOS expres- sion by macrophages was examined. We show that EMCV-in-duced iNOS expression and nitrite production by RAW264.7 cells and primary macrophages are more sensitive to inhibition by (S)-BEL than (R)-BEL. Furthermore, EMCV-induced iPLA₂ ac- tivity and the downstream phosphorylation and activation of the MAPK response element-binding protein (CREB), a transcription factor essential for virus-induced iNOS expression, are attenu- ated by (S)-BEL but largely unaffected by the presence of (R)- BEL at similar concentrations. Using a genetic approach to con- firm these findings, we show that EMCV-induced iNOS expression and nitrite production are abolished in iPLA₂β⁻/⁻ macrophages as compared with wild-type macrophages. These studies support a primary role for iPLA₂β in the regulation of virus-induced iNOS expression by macrophages.

EXPERIMENTAL PROCEDURES

Materials and Animals—EMCV was the generous gift of Dr. Ji-Won Yoon (University of Calgary, Calgary, Alberta, Canada) and grown and maintained as previously described (8). Poly(I:C) was purchased from Sigma and prepared as previously described (23). The racemic mixture of BEL was obtained from Cayman Chemicals (Ann Arbor, MI), and (R)- and (S)-BEL were the generous gift of Dr. Richard Gross (Washington University, St. Louis, MO) and prepared as previously described (22). PCR primers were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). Rabbit anti-iNOS antiserum was a gift from Dr. Pam Manning (Pfizer, St. Louis, MO), rabbit anti-phospho-CREB antiserum was obtained from Upstate USA, Inc. (Charlottesville, VA), rabbit anti-ERK2 was a gift from Dr. John C. Lawrence, Jr. (University of Virginia, Charlottesville, VA), rabbit anti-STAT was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), 3ZD monoclonal mouse anti-IL-1β was obtained from Biological Resources Branch, Division of Cancer Treat- ment, Diagnosis at the National Cancer Institute, and mouse anti- GAPDH antiserum was purchased from Ambion (Austin, TX). C57BL/ 6J- SV129 mice were obtained from The Jackson Laboratories (Bar Harbor, ME). The generation and maintenance of iPLA₂β⁻/⁻ and iPLA₂β+/⁻ mice have been previously described and were generously provided by Dr. John Turk (Washington University, St. Louis, MO) (24). The St. Louis University Institutional Animal Care and Use Committee has approved all animal studies contained in this report.

Cell Culture, Isolation, and Viral Infection—RAW264.7 cells were removed from growth flasks by treatment with 0.05% trypsin/0.02% EDTA for 5 min at 37 °C. Primary mouse macrophages were isolated by peritoneal lavage as previously described (25). Following isolation, the cells were washed, plated at 4 × 10⁵ cells/400 μl of Complete CMRL-1086 as previously described (23), and allowed to adhere for 2 h prior to use. Aliquots of experiments, RAW264.7 cells or primary macrophages (4 × 10⁵ cells/400 μl DEMEM) were precultured for 30 min at 37 °C with the indicated concentrations of racemic BEL or R- or S- enantiomers, followed by the addition of EMCV (1 μl/μl cell culture) or poly(C) (50 μg/ml), and continued in culture for 0.5, 6, or 24 additional h as indicated in the figure legends.

RT-PCR and Quantitative PCR Analysis—Total RNA was isolated from RAW264.7 cells using the RNeasy kit (Qiagen, Santa Clarita, CA) according to the manufacturer’s instructions and used to prepare a first-strand cDNA library using the Superscript Pre-amplification System from Invitrogen. Standard PCR was performed using iPLA₂β, iPLA₂γ, iNOS, and GAPDH oligonucleotide primers as previously de- scribed (26). Quantitative real-time PCR was performed using the Quantitect SYBR Green PCR kit (Qiagen) according to the manufac- turer’s instructions, and iNOS mRNA levels were quantified and nor- malized to GAPDH. iPLA₂β-specific primers were 5′-GCTATTACCA-GCTTCATC3′ (forward) and 5′-GAGAGTTTCTTCACCTTGTTG-3′ (reverse), and iPLA₂γ-specific primers were 5′-CAGTTTCAAGGGTAT- TTTTGG-3′ (forward) and 5′-TTTTTTCTCCGCTTTGGAATAAG-3′ (reverse). iNOS and GAPDH primers have been described previously (26), and GAPDH was used as an internal control for all PCRs.

Nitrite Formation—Nitrite production was determined by the addition of 50 μl of Greiss reagent to 50 μl of cell culture supernatant and absorbance at 540 nm compared with a sodium nitrite standard curve (27). The activity of iPLA₂βγ was examined as previously described (7). In brief, macrophages (6 × 10⁶) were washed twice with phosphate-buffered saline, resuspended in 400 μl of ice-cold PL-supernatant, 10 μM iPLA₂βγ, 10 mM 3-isobutyl-1-methylxanthine (IBMX), 5 μM EDTA, and 2 μM dithiothreitol, and lysed by sonication at 4 °C. The cell lysate was centrifuged at 14,000 × g for 20 min at 4 °C, and the supernatant fraction was further centrifuged at 100,000 × g for 60 min. The membrane pellet was suspended in PL-supernatant, and the absorbance at 540 nm was determined. The iPLA₂βγ activity was measured in the presence of 2 μg/ml of unlabelled fatty acid and [3H]arachidonic acid. iPLA₂βγ activity was determined by two methods: the first was a fluorometric assay and the second was a radiometric assay. The fluorometric assay was performed on a fluorimeter (Molecular Probes) using a Perkin-Elmer Life Sciences spectrophotometer. The radiometric assay was performed using a scintillation spectrometer (Model 1900; Packard Instrument Co.) and a 96-well plate reader (MicroWell; Bio-Tek Instruments). The data were analyzed with the program Enzyme Kinetics (St. Louis, MO) using a non-linear least-squares method.

Western Blot Analysis—Western blot analysis was performed as pre- viously described (29). Antibody dilutions were as follows: rabbit anti-iNOS, 1:1000; rabbit anti-STAT, 1:1000; rabbit anti-phospho-CREB, 1:1000; rabbit anti-ERK2, 1:1000; mouse anti-IL-1β, 1:2000; mouse anti-GAPDH, 1:5000; horseradish peroxidase-conjugated donkey anti-rabbit, 1:5000; and horseradish peroxidase-conjugated donkey anti-mouse, 1:5000.

Densitometry and Statistical Analysis—Autoradiograms were scanned into National Institutes of Health Image Version 1.59 using a
Virus-induced iNOS Expression Requires iPLA₂β

Macrophages Express iPLA₂β and iPLA₂γ—Virus infection has been reported to increase membrane phospholipid metabolism by accelerated phospholipid catabolism, although the specific enzymes participating in the cellular response to viral infection were not known with certainty (30, 31). Consistent with these early reports, we have shown that treatment of macrophages with the viral dsRNA mimetic poly(IC) resulted in a 4-fold increase in membrane-associated iPLA₂ activity that is sensitive to inhibition by BEL. In a similar concentration-related manner, BEL also attenuates poly(IC)- and EMCV-induced iNOS expression by macrophages (7). Whereas these studies provide evidence to support a role for iPLA₂ in the antiviral response of iNOS expression by macrophages, the specific isoform of iPLA₂ activated by virus infection that contributes to the transcriptional regulation of iNOS has yet to be established. Two mammalian forms of iPLA₂ have been characterized, and both are expressed in macrophages under basal conditions and following a 6-h infection with EMCV as determined by RT-PCR (Fig. 1). Similar to RAW264.7 cells, peritoneal macrophages express both isoforms of iPLA₂, and EMCV infection fails to modulate the expression of either isoform (data not shown). To confirm that the iPLA₂γ PCR product was specific and not due to amplification of a homologous sequence of iPLA₂β cDNA, an iPLA₂β-encoding plasmid (iPLA₂β-pDONR) was used as the DNA template, and primers for iPLA₂β but not iPLA₂γ produced a PCR product of the predicted size. These findings indicate that the iPLA₂γ PCR product is specific and not due to mispriming or erroneous amplification of iPLA₂β cDNA. In addition, sequence analysis was used to confirm the identity of each PCR product (data not shown). These results indicate that macrophages express both iPLA₂β and iPLA₂γ and suggest that either isoform could potentially participate in the regulation of virus-induced iNOS expression.

(S)-BEL Inhibits EMCV-induced iNOS Expression and Nitrite Production by Macrophages—We have recently shown that treatment of macrophages with the viral dsRNA mimetic poly(IC) or infection with EMCV results in iNOS expression and nitrite production, events that are inhibited in a concentration-related manner by the iPLA₂ inhibitors BEL and arachidonoyl trifluoromethyl ketone (7). Until recently, it was impossible to distinguish biological effects mediated by an individual isoform of iPLA₂ pharmacologically using BEL because the racemic mixture inhibits both iPLA₂β and iPLA₂γ enzymatic activity at low micromolar concentrations (15, 20). The (R)- and (S)-enantiomers of BEL have, however, recently been shown to selectively inhibit individual iPLA₂ isoforms in a narrow concentration range (22). Because the selectivity of these enantiomers for iPLA₂β and iPLA₂γ is not absolute and is decidedly dependent upon concentration, we used submaximally effective concentrations of (R)-BEL and (S)-BEL to determine their differential effects on virus-induced iNOS expression by macrophages. Because EMCV infection induces membrane-associated iPLA₂ enzymatic activity, and iPLA₂γ resides primarily in peroxisomes and mitochondria (15), we postulated that iPLA₂γ may participate in the antiviral response and that virus-induced iNOS expression would be inhibited by (R)-BEL. To examine this possibility, the inhibitory actions of racemic BEL, as well as the (R)- and (S)-enantiomers of BEL, on EMCV-induced nitric oxide production (Fig. 2A) and iNOS expression (Fig. 2B) were examined. Racemic BEL and (S)-BEL inhibit EMCV-induced nitrite production and iNOS expression by RAW264.7 cells in a similar concentration-related manner with >70% inhibition at 5 μM. In contrast, (R)-BEL is much less effective at inhibiting EMCV-induced nitrite production and iNOS expression, with <40% inhibition at 5 μM. Similar to RAW264.7 cells, racemic BEL and (S)-BEL inhibit EMCV + IFN-γ-induced nitrite production and iNOS expression by primary macrophages to similar levels, whereas (R)-BEL fails to significantly inhibit nitric oxide production and iNOS expression (Fig. 3). Alone, (R)-BEL, (S)-BEL, or racemic BEL does not modulate basal nitrite production by RAW264.7 cells or primary mouse macrophages (data not shown). Because (S)-BEL is 10-fold more selective for iPLA₂β than iPLA₂γ, and (S)-BEL is significantly more effective at inhibiting EMCV- and EMCV + IFN-γ-induced nitrite production and iNOS expression by macrophages than (R)-BEL (Figs. 2 and 3), these findings suggest that iPLA₂β is likely the predominant isom
that participates in the regulation of iNOS expression by macrophages.

(S)-BEL Inhibits dsRNA-induced iPLA2 Enzymatic Activity—iPLA2 has historically been ascribed a homeostatic role in membrane phospholipid maintenance, and thus there is a basal level of iPLA2 enzymatic activity present in unstimulated macrophages. However, in previous studies, we have shown that dsRNA stimulates a membrane-associated phospholipase activity in macrophages that is inhibited by racemic BEL at concentrations (1–5 μM) that also inhibit iNOS expression (7). Consistent with these previous findings, treatment of macrophages for 30 min with the viral mimetic poly(IC) results in a 3-fold increase in membrane-associated iPLA2 activity, and this activation is sensitive to inhibition by racemic BEL and (S)-BEL at a concentration of 5 μM (Fig. 4). At this concentration, racemic BEL and (S)-BEL attenuate dsRNA- and EMCV-induced iNOS expression and nitric oxide production (Fig. 1). However, the iPLA2γ-selective (R)-BEL fails to attenuate dsRNA-stimulated iPLA2 activity at a concentration of 5 μM. In a similar manner, racemic BEL and (S)-BEL, but not (R)-BEL, inhibit membrane-associated iPLA2 activity in EMCV-infected macrophages (data not shown). These data suggest that iPLA2β is the isoform activated in macrophages during the antiviral response.

(S)-BEL Inhibits iPLA2-mediated Signaling and CREB Activation—We have previously identified the downstream targets of iPLA2 that participate in the transcriptional regulation of iNOS expression in response to virus infection, particularly protein kinase A (PKA) and the subsequent phosphorylation and activation of the transcription factor CREB (7). Furthermore, we have shown that EMCV infection of RAW264.7 cells and primary mouse macrophages induces the rapid and transient phosphorylation of CREB at 15 and 30 min post-infection (32). As shown in Fig. 5, infection of RAW264.7 cells with EMCV results in CREB phosphorylation within 30 min. Racemic BEL and (S)-BEL, but not (R)-BEL, attenuate EMCV-induced CREB phosphorylation (Fig. 5B). The inhibitory actions of (S)-BEL on EMCV-induced CREB phosphorylation are consistent with the effects of this iPLA2β-selective inhibitor on poly(IC)- and EMCV-induced iNOS expression and provide further evidence to support a role for iPLA2β in the regulation of iNOS expression by macrophages in response to virus infection.

EMCV-induced iNOS Expression Is Attenuated in iPLA2β−/− Macrophages—As we have used the R- and S-enantiomers of BEL to provide pharmacological evidence consistent with a role for iPLA2β in the regulation of virus-induced iNOS expression by macrophages, we used a genetic approach to further support our findings. To confirm that iPLA2β participates in this antiviral response, the effects of EMCV infection on iNOS expression by macrophages isolated from iPLA2β−/− mice were evaluated. In the presence of IFN-γ, EMCV infection of wild-type macrophages stimulates iNOS protein expression by 24 h (Fig. 6A), which is preceded by mRNA accumulation observed by 6 h post-infection (Fig. 6, B and C). Neither EMCV nor IFN-γ alone is sufficient to induce iNOS expression, which is consistent with a requirement of two proinflammatory signals for the transcriptional activation of iNOS in primary macrophages (23). In contrast, the combination of EMCV + IFN-γ fails to stimulate iNOS protein expression (Fig. 6A) and mRNA accumulation (Fig. 6, B and C) by iPLA2β−/− macrophages. The defect in virus-induced iNOS expression by iPLA2β−/− macrophages is selective for iNOS and does not appear to be associated with a complete inhibition of the antiviral response because EMCV + IFN-γ stimulate IL-1β expression to similar levels in macrophages isolated from wild-type and iPLA2β−/− mice (Fig. 6D). These findings are consistent with previous work in which we showed that BEL has no inhibitory effect on dsRNA-induced IL-1 expression by macrophages (7). These data provide genetic evidence to further support our findings using BEL enantiomers and suggest a role for iPLA2β in the regulation of virus-induced iNOS expression by macrophages.

DISCUSSION

PKR is a central regulator of numerous cellular responses during viral infection, including activation of inflammatory gene expression, inhibition of protein translation, and induction of apoptosis. Recently, a number of PKR-independent pathways have been identified that participate in the regulation of transcription factors and inflammatory gene expression. Studies have provided evidence that dsRNA and EMCV acti-
vate NF-κB to similar levels in both PKR+/+ and PKR−/− mouse embryonic fibroblasts and macrophages (4, 23). Additional transcription factors that participate in the antiviral response may also be independent of PKR because the dsRNA-induced activation of interferon regulatory factor-1 in macrophages does not require the presence of functional PKR (33). Furthermore, PKR is not required for virus-induced transcriptional activation of the inflammatory genes IL-1, cyclooxygenase-2, and iNOS because EMCV and dsRNA stimulate the expression of these genes to similar levels in mouse macrophages isolated from either PKR+/+ or PKR−/− mice (6, 23). Although NF-κB activation is required, alone it is insufficient to stimulate the transcriptional activation of these inflammatory mediators. EMCV- or dsRNA-induced activation of these inflammatory genes requires a second signal transduction cascade, which appears selective for individual target genes. We have shown that dsRNA and EMCV stimulate IL-1 expression in an ERK-dependent manner (5), cyclooxygenase-2 expression requires the activation of c-Jun NH2-terminal kinase and p38 (34), and iPLA2 activation is essential for iNOS expression (7).

The aim of the current study was to further elucidate the role of iPLA2 in the regulation of iNOS expression by macrophages in response to virus infection. In previous studies, the iPLA2β-selective inhibitor BEL was shown to inhibit EMCV- and dsRNA-induced iNOS expression in a concentration-related manner that correlated with the inhibition of iPLA2 enzymatic activity. We have identified PKA as one downstream target of iPLA2 by phosphorylating and activating the transcription factor CREB. Evidence to support this novel lipid-mediated signal transduction pathway in macrophages includes 1) the inhibition of dsRNA-induced iNOS expression by inhibitors of PKA, 2) the ability of direct PKA activation (using non-hydrolyzable analogues of cAMP) to overcome the inhibitory actions of BEL on dsRNA-induced iNOS expression, 3) dsRNA stimulation of CREB phosphorylation and CREB-dependent reporter activity in a BEL-sensitive manner, and 4) the observation that mutations in the proximal and distal cAMP response elements of the iNOS promoter result in near complete inhibition of EMCV-induced iNOS reporter activity. Furthermore, exogenous addition of an analogue of lysoplasmencylcholine, a product of iPLA2 activity, reconstitutes dsRNA-induced iNOS expression in the presence of BEL. These findings are consistent with the iPLA2-dependent phosphorylation of CREB following 5 min of global ischemia in adult rat hearts (35) as well as the ability of lysoplasmencylcholine generated by iPLA2 to stimulate CREB phosphorylation in endothelial cells (36).

BEL is an inhibitor of the group VI phospholipases (20). The two mammalian isoforms in this class of phospholipases are iPLA2β and iPLA2γ. Because BEL inhibits both isoforms at low micromolar concentrations, it is impossible to attribute a biological response to a specific iPLA2 isoform based on inhibition by racemic BEL. Recently, Jenkins et al. (22) used chiral high pressure liquid chromatography to separate and purify the R- and S-enantiomers of BEL and subsequently demonstrated that (S)-BEL is 1 order of magnitude more selective for iPLA2β than iPLA2γ. We have examined the specific roles of iPLA2β and iPLA2γ in the regulation of iNOS expression by macrophages in response to EMCV infection using these BEL enantiomers. Whereas mouse macrophages express both mammalian isoforms of iPLA2, the iPLA2β-selective (S)-BEL is 10-fold more effective at inhibiting EMCV-induced iNOS expression, nitrite production, and CREB phosphorylation as compared with (R)-BEL. Consistent with the inhibition of iNOS expression, (S)-BEL and racemic BEL inhibit dsRNA-induced iPLA2β activity in RAW264.7 cells, whereas at the same concentration, (R)-BEL fails to inhibit iPLA2γ activity. The inability of (R)-BEL to inhibit iPLA2γ enzymatic activity suggests that iPLA2β, but not iPLA2γ, is activated in response to dsRNA and that iPLA2β selectively regulates the expression of iNOS. Consistent with this interpretation, EMCV-induced iNOS mRNA accumulation and protein expression are completely abrogated in mice specifically deficient in iPLA2β, despite the presence of iPLA2γ. Taken together, these findings indicate that the participation of individual iPLA2 isoforms in the antiviral response of iNOS expression is neither redundant nor compensatory such that iPLA2β appears to be selectively activated in response to treatment with dsRNA or following virus infection of macrophages.

Whereas our data consistently display a differential sensitivity of virus-induced iNOS expression, nitrite production, CREB phosphorylation, and iPLA2 enzymatic activity to BEL enantiomers, we failed to completely inhibit these events with BEL at concentrations of 5 μM. Given that BEL is not a saturating inhibitor but rather a mechanism-based inhibitor that is only effective when iPLA2 is in an active form, a portion of the BEL-insensitive component of this antiviral response is likely due to competition with exogenous phospholipids or inaccessibility due to enzyme compartmentalization. The apparent BEL insensitivity also derives, in part, from the fact that submaximally effective concentrations of BEL were used, and these concentrations were carefully chosen because it is only in this narrow concentration range that one sees differential effects of the inhibitors. Only at these concentrations is it possible to make meaningful observations about the participation of individual iPLA2 isoforms to a given biological response. At a sufficiently high concentration, it is not possible to use inhibition by the individual R- and S-enantiomers of BEL to determine effects mediated by individual iPLA2 isoforms because inhibition of iPLA2β and iPLA2γ by (R)- and (S)-BEL is not absolute. In addition, the ability of racemic BEL to inhibit virus-induced iNOS expression and nitrite production to levels

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*S. A. Steer and J. A. Corbett, unpublished data.*
similar to (S)-BEL reflects that, at most, there is a theoretical 1.6-fold difference between potencies of racemic BEL and (S)-BEL because low concentrations of (R)-BEL inhibit the activity of iPLA₂β by ~30% (22).

Although BEL is a selective inhibitor of iPLA₂, it has been shown to inhibit another enzyme involved in phospholipid metabolism, phosphatidate phosphohydrolase-1, thus caution must be exercised when assigning biological activities to iPLA₂ when studies are conducted solely using BEL. To substantiate our findings using pharmacological inhibitors and to confirm that iPLA₂ is a primary regulator of virus-stimulated iNOS expression, we examined the effects of EMCV infection on iNOS expression in macrophages isolated from the recently generated iPLA₂β-deficient mice (24). Consistent with our pharmacological studies using the R- and S-enantiomers of BEL, EMCV-induced iNOS expression is abolished in iPLA₂β-deficient macrophages, whereas EMCV stimulates robust iNOS expression in wild-type controls. Furthermore, the role of iPLA₂β in the regulation of inflammatory gene expression in response to virus infection appears to be selective for iNOS because EMCV stimulates IL-1β expression to similar levels in macrophages isolated from iPLA₂β-/- or iPLA₂β+/+ mice. These findings provide genetic evidence to further support our pharmacological studies and suggest a requirement for iPLA₂β in the transcriptional regulation of iNOS expression by macrophages during viral infection.

Whereas these results are consistent with a role for iPLA₂β in cell signaling, they also address the role of iPLA₂ in the regulation of normal macrophage function. Previous studies have suggested that the primary role for iPLA₂ in macrophages is the regulation of lipid homeostasis, to provide lysophospholipids for the hydrolysis of membrane phospholipids (16). Our findings demonstrate that iPLA₂β functions in macrophage intracellular signaling by providing products that activate kinases and transcription factors such as PKA and CREB (7).

Although we have implicated the involvement of iPLA₂β in the antiviral response and characterized its participation in a novel lipid signaling pathway that regulates virus-induced iNOS expression by macrophages, we have yet to identify the mechanism by which virus infection stimulates iPLA₂β enzymatic activity. Interestingly, the iPLA₂β-dependent regulation of iNOS expression in response to viral infection does not require the classical mediator of antiviral activities in infected cells, PKR, thus implicating iPLA₂β as an additional PKR-independent signaling pathway that participates in the antiviral response. Furthermore, iPLA₂β and NF-κB activation, both of which are required for iNOS expression, appear to be regulated by independent mechanisms because siRNA and EMCV are equally effective at stimulating NF-κB activity in the presence of concentrations of BEL that prevent iNOS expression (7). Recently, we have obtained evidence that activation of signaling cascades that participate in the regulation of inflammatory gene expression by macrophages is receptor-mediated because EMCV infection activates iPLA₂, NF-κB, CREB, and mitogen-activated protein kinases within 15 min. Under these conditions, viral RNA accumulation and protein expression, early indicators of viral infection, are undetectable within infected macrophages. Additionally, treatment of macrophages with empty virions (capsid structures void of viral RNA) results in the time-dependent activation of iPLA₂, NF-κB, CREB, and mitogen-activated protein kinase in a manner similar to wild-type virus (32). The canonical receptors targeted by EMCV to facilitate cell attachment and entry are members of the cell adhesion molecule superfamily, and vascular cell adhesion molecule has been identified as a receptor for EMCV on vascular endothelial cells (37). We are currently investigating the involvement of this family of receptors in the activation of iPLA₂β during virus infection of macrophages.

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