Glycogen synthase kinase-3β negatively regulates group IIA phospholipase A₂ expression in human aortic smooth muscle and HepG2 hepatoma cells

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Abstract **The present study shows that the IFN-γ-mediated upregulation of secretory phospholipase A₂ of group IIA (sPLA₂-IIA) in HASMC and HepG2 cells is synergistically increased after simultaneous inhibition of glycogen synthase kinase-3β (GSK-3β) by indirubin-3′-monoxime, 5-iodo or AR-A014418. The effect of GSK-3β inhibition was dose- and time-dependent and can be further augmented by its concomitant incubation with *Clostridium difficile* toxin B, an inhibitor of small Rho proteins, or H-1152, an inhibitor of Rho-associated kinase. Using AG-490 and caffeic acid phenethyl ester (CAPE), it is further demonstrated that the effect of GSK-3β inhibition on sPLA₂-IIA expression depends on Janus kinase-2 and NF-κB-signaling.**
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Keywords: Secretory phospholipase A₂; GSK-3β; Jak/STAT; NF-κB; HASMC; HepG2 cell

1. Introduction

The secretory group IIA phospholipase A₂ is constitutively expressed in a number of mammalian cell types and tissues. In hepatic, endothelial, mesangial, and smooth muscle cells, secretory phospholipase A₂ of group IIA (sPLA₂-IIA) is induced in vitro upon stimulation with proinflammatory cytokines (for review [1]). Increased serum activities of this enzyme can be observed in severe acute inflammatory diseases such as sepsis, septic shock [2,3], acute pancreatitis [4], and multiple injuries [5,6]. The origin of the serum sPLA₂-IIA continues to be a matter of some controversy. Along with activated leukocytes, smooth muscle cells and the liver come particularly into question as a locale for synthesis [7]. The detailed function of the raising sPLA₂-IIA levels in serum during inflammation is also still unspecified. Similar to other phospholipases A₂, sPLA₂-IIA may be involved in cell signaling, apoptosis, remodeling of cell membranes, inflammatory response, and host defense against bacterial invaders (for review [8,9]). Apart from direct effects through phospholipolysis, sPLA₂-IIA can also elicit proinflammatory changes in an autocrine manner by the induction of tumor necrosis factor-receptor superfamily signaling and activation of MAP kinase cascade [10,11]. Thus, the knowledge of mechanisms regulating the expression of sPLA₂-IIA is of special importance.

It is established that the expression of sPLA₂-IIA is cell- and species-specific [1,12] and depends on the activity of cellular signaling pathways and transcription factors such as nuclear factor-kappa B (NF-κB), CCAAT box enhancer binding proteins (C/EBP), Janus tyrosine kinase (Jak)/signal transducer and activator of transcription (STAT), phosphatidylinositol-3-kinase-Akt, peroxisome proliferator-activated receptor, protein kinase C, cAMP/protein kinase A, and mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK) [13–17]. Recently, it was shown that interleukin-1β (IL-1β)-stimulated sPLA₂-IIA expression in rat mesanglial cells is negatively controlled by RhoA/Rho-kinase pathways [18]. We have also observed a similar negative control of interferon-γ (IFN-γ)-mediated sPLA₂-IIA expression through RhoA/Rho-kinase and Ras/Raf/MEK/ERK pathways in human aortic smooth muscle cells (HASMC) and HepG2 (submitted for publication).

In the present study, we pursued the question to what extent glycogen synthase kinase-3β (GSK-3β) is involved in this negative regulation of sPLA₂-IIA expression. It is known that the activity of GSK-3β is increased after Tyr-216 phosphorylation through RhoA/Rho-kinase [19] and MEK1/2 signaling [20]. We demonstrate for the first time that the activity of GSK-3β is important in the regulation of sPLA₂-IIA and that inhibition of GSK-3β results in a potentiation of IFN-γ-induced sPLA₂-IIA expression in HASMC and HepG2 cells via processes which are Jak2 and NF-κB-dependent.

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2. Materials and methods

2.1. Materials

Recombinant human IFN-γ was purchased from R&D Systems GmbH (Wiesbaden-Nordenstadt, Germany). *Clostridium difficile* toxin B (TcdB), indirubin-3′-monoxime, 5-iodo-, AR-A014418 (*N*-(4-methoxybenzyl)-*N*′-(5-nitro-1,3-thiazol-2-yl)urea), AG-490 (Tyrphostin B42), and H-1152 ((S)-(+)-2-methyl-1-[(4-methyl-5-isoquinolinyl)-sulfonyl]homopiperazine, 2HCl) were from Calbiochem (San Diego, California, USA). Caffeic acid phenethyl ester (CAPE) was purchased from Sigma–Aldrich. Polyclonal rabbit antibodies against sPLA$_2$-IIA were obtained from Cayman Chemical (MI, USA). AR-A014418, indirubin-3′-monoxime, 5-iodo-, AG-490, and CAPE were dissolved in dimethyl sulfoxide (DMSO). The final concentrations of solvents were 0.3% or less. Controls using DMSO alone were run in all cases.

2.2. Cell culture and incubation

HASMC were purchased from Promocell (Heidelberg, Germany) and cultured according to the instructions in smooth muscle cell growth medium-2 at 37 °C in a humidified atmosphere of 5% CO$_2$. The human HepG2 hepatoma cell line was obtained from American Type Culture Collection (Rockville, Maryland, USA) and maintained in RPMI supplemented with 10% fetal calf serum (FCS) and cultured under the same condition as described for HASMC.

For RNA isolation and determination of sPLA$_2$-IIA protein in cells released into the media, cells were plated onto 6-well and 24-well plates at a cell density of 10^4 cells/cm^2. For all experiments, exponentially growing subconfluent HASMC and confluent HepG2 cells were used at passages 5–8. At the end of the incubation period, media were removed, centrifuged with $800 \times g$ for 10 min to remove cell debris, and kept at −80 °C until analysis for sPLA$_2$-IIA concentration. The attached cells were lysed in TRI Reagent (Sigma–Aldrich, Deisenhofen, Germany) and stored at −80 °C until RNA isolation or lysed in cell lysis buffer (Promega) after washing with phosphate-buffered saline and stored at −80 °C until analysis for sPLA$_2$-IIA content.

2.3. RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

RNA was isolated after lysis of cells in TRI reagent according to the manufacturer's instructions. Isolated RNA was converted to cDNA by using the GeneAmp RNA-PCR Kit (Perkin–Elmer). A portion of the RT reaction products was then amplified for identification of sPLA$_2$-IIA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as house-keeping gene using PCR. The applied primer pairs were 5′-GTGATCATGATCTTTGGCCTACTGCA-3′ and 5′-TCTCCCTCGTGGGGAGCAACGACT-3′ for sPLA$_2$-IIA, giving PCR products with a length of 411 bp, and 5′-CGGAGT-CAACGGATTTGGTCGTATTG-3′ and 5′-GCAGGAGGCATTG-C-TGATGATCTTG-3′ for GAPDH amplifying products with 439 bp length. The oligonucleotides for the analysis of sPLA$_2$-IIA mRNA were synthesized according to the published nucleotide sequence of human placental sPLA$_2$-IIA cDNA [21] and for GAPDH-mRNA according to the published nucleotide sequence [22].

2.4. Quantitative determination of sPLA$_2$-IIA by ELISA

The sPLA$_2$-IIA protein in cells released into the medium was determined using specific enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (Cayman Chemical, MI). Total cell protein was determined by using Bicinchoninic Acid assay kit with bovine serum albumin as internal standard (Sigma–Aldrich).

2.5. Western blot analysis

Aliquots of the medium were incubated with Laemmli buffer at 95 °C for 5 min and the proteins were separated by 7.5%ic SDS polyacrylamide gel electrophoresis. After transferring of proteins to nitrocellulose membranes by electroblotting, the membranes were incubated with a specific polyclonal rabbit anti-sPLA$_2$-IIA antiserum overnight at 4 °C. After washing, the nitrocellulose membranes were incubated with horseradish peroxidase-labeled anti-rabbit IgG for 1 h at room temperature. The sPLA$_2$-IIA protein was visualized by enhanced chemiluminescence using the ECL system (Amersham Pharmacia Biotech, Freiburg, Germany).
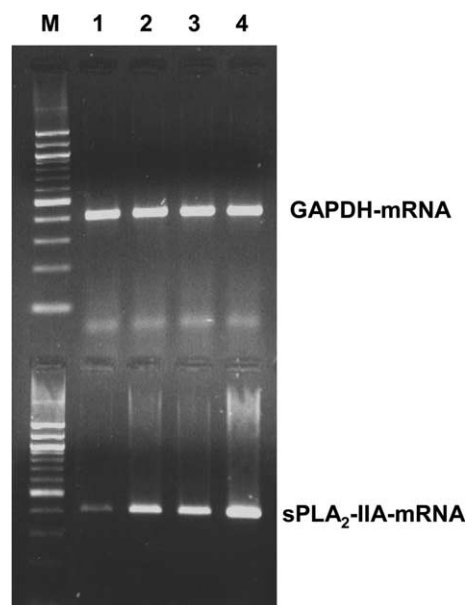

Fig. 1. Upregulation of sPLA$_2$-IIA mRNA in HASMC by indirubin-3′-monoxime,5-iodo (indirubin). After exposure of HASMC for 48 h to indirubin and IFN-γ, both alone and in combination, the sPLA$_2$-IIA mRNA in relation to GAPDH mRNA was determined by RT-PCR. Agarose gel electrophoresis with ethidium bromide-stained amplificates is shown. M indicates 100 bp molecular weight ladder; lane 1, control; lane 2, 50 nM indirubin; lane 3, 25 ng/ml IFN-γ; lane 4, 50 nM indirubin and 25 ng/ml IFN-γ.

3. Results

3.1. Upregulation of sPLA$_2$-IIA expression in HASMC by GSK-3β inhibition

Fig. 1 shows the effect of indirubin-3′-monoxime,5-iodo (50 nM) alone and in combination with IFN-γ (25 ng/ml) on the level of sPLA$_2$-IIA mRNA in HASMC. Here, after incubation of the cells with both IFN-γ and indirubin, an increase of sPLA$_2$-IIA mRNA could be observed (Fig. 1, lanes 2 and 3). When both agents were added simultaneously to the cell medium, a further increase in sPLA$_2$-IIA mRNA was the result (Fig. 1, lane 4). These data agreed with the sPLA$_2$-IIA protein quantities secreted from the HASMC into the medium and obtained by ELISA. Thus, in comparison to the control cells the sPLA$_2$-IIA secretion increased 4-fold after incubation for 48 h with 25 ng/ml IFN-γ and 3-fold with 50 nM indirubin (Fig. 2). When indirubin was introduced in combination with IFN-γ in equal concentrations, the secretion of sPLA$_2$-IIA increased 25-fold, indicating a synergistic effect of both components. A similar synergism arose, albeit not as pronounced, between IFN-γ and AR-A014418 as well (Fig. 2). When the cells were stimulated with 10 ng/ml TcdB as an inhibitor of the Rho proteins and 25 ng/ml IFN-γ, the sPLA$_2$-IIA secretion climbed to a level 20 times that of the control cells. The combination of TcdB (10 ng/ml) and IFN-γ (25 ng/ml) with indirubin (50nM) led finally to an increase in sPLA$_2$-IIA secretion of more than 100 times. In this instance, the increase was dependent on the concentration of indirubin (Fig. 2). In this fashion, the sPLA$_2$-IIA protein secretion increased to approximately 4–15 times that of the control cells when indirubin in concentrations ranging from 12.5 to 200 nM was
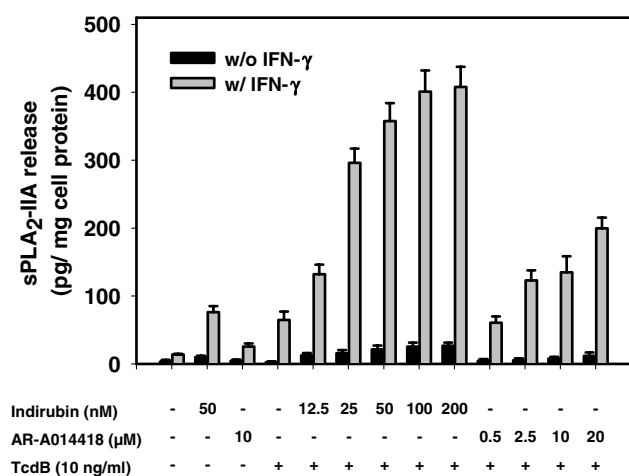
Fig. 2. Dose-dependent increase of sPLA$_2$-IIA secretion in HASMC by GSK-3β inhibitors, indirubin and AR-A014418, alone and in combination with IFN-γ and TcdB. After exposure of cells to GSK-3β inhibitors in varied concentrations for 48 h in the absence or presence of 25 ng/ml IFN-γ and 10 ng/ml TcdB, the sPLA$_2$-IIA protein released into the medium was determined. Results are means ± S.D. of three samples per experiment and are representatives of three independent experiments.

introduced into the cell medium simultaneously with 10 ng/ml TcdB (Fig. 2). A 40- to 120-fold increase in comparison to the control cells resulted when, along with indirubin (12.5–200.0 nM) and 10 ng/ml TcdB, 25 ng/ml IFN-γ was added simultaneously (Fig. 2). A similar, concentration-dependent increase could be observed following incubation of the cells with AR-A014418. Along with the dependence on concentration, the effects of indirubin were time-dependent (Fig. 3). Similar results apply to AR-A014418 (data not shown).

3.2. Upregulation of sPLA$_2$-IIA expression in HepG2 cells by GSK-3β inhibition

To verify the cell specificity of GSK-3β-dependent sPLA$_2$-IIA upregulation, human HepG2 hepatoma cells were exposed to indirubin alone and in the presence of 25 ng/ml
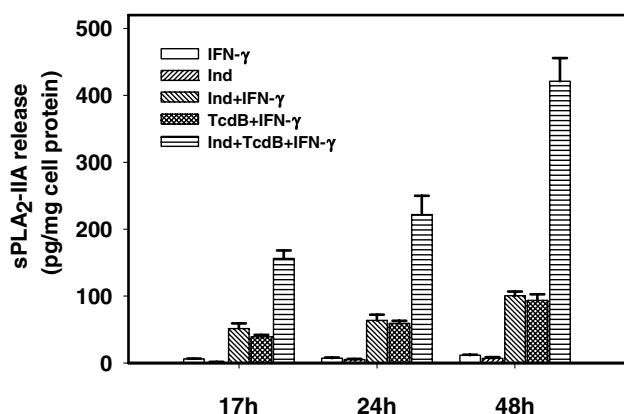

Fig. 3. Time course of sPLA$_2$-IIA secretion in HASMC incubated with indirubin alone and in combination with IFN-γ and TcdB. After treatment of HASMC for 17–48 h with 50 nM indirubin in the absence or presence of 25 ng/ml IFN-γ and 10 ng/ml TcdB, the sPLA$_2$-IIA protein released into the cell medium was determined. Results are means ± S.D. of three samples per experiment and are representatives of three independent experiments.

IFN-γ or in combination with H-1152, a Rho-kinase inhibitor (Fig. 4). Similar to the HASMC, a significant upregulation of sPLA$_2$-IIA expression in HepG2 cells was observed by stimulation with IFN-γ. The incubation of the cells with H-1152 also led to a significant increase in sPLA$_2$-IIA secretion, while incubation with indirubin alone produced only a minimal increase. When indirubin was combined with IFN-γ, the sPLA$_2$-IIA secretion rates increased significantly, indeed to a lesser extent when compared to those observed after incubation of HASMC with indirubin and IFN-γ. The lower observed level of sPLA$_2$-IIA induction could possibly be explained by the relatively high basal level of sPLA$_2$-IIA secretion of HepG2 cells, which was approximately 800 times as high as those of HASMC. The strongest increase in sPLA$_2$-IIA secretion was reached when the HepG2 cells were incubated simultaneously with indirubin, H-1152, and IFN-γ (Fig. 4).

3.3. Inhibition of Jak2/STAT and NF-κB signaling prevents sPLA$_2$-IIA upregulation in HASMC and HepG2 cells

In an earlier study, we found that in the negative regulation of sPLA$_2$-IIA expression through RhoA/Rho-kinase and Ras/Raf/MEK/ERK pathways, Jak2- and NF-κB-dependent pathways play a significant role. Thus, we applied inhibitors of Jak2- (AG-490) and NF-κB-signaling (CAPE) to examine whether these pathways could also be involved in the GSK-3β-controlled expression of sPLA$_2$-IIA. Here, it could be shown that both AG-490 and CAPE were able to reduce the level of sPLA$_2$-IIA transcripts and to block almost completely the synthesis and secretion of sPLA$_2$-IIA in HASMC, induced through incubation with (i) indirubin and IFN-γ alone; (ii) indirubin and IFN-γ in combination with TcdB; and (iii) AR-A014418, IFN-γ, and TcdB (Fig. 5). Similar results were obtained after adding pyrrolidine dithiocarbamate (PTDC) and 20 S Proteasome inhibitor I (PSI) as further inhibitors of NF-κB-signaling and in HepG2 cells (results not shown).

4. Discussion

To our knowledge, this is the first report showing the importance of GSK-3β activity in the regulation of sPLA$_2$-IIA expression in HASMC and HepG2 cells. Indeed, sPLA$_2$-IIA expression either remained unchanged or increased only slightly as a result of the inhibition of GSK-3β alone. However, a large increase in sPLA$_2$-IIA expression could be demonstrated when IFN-γ was simultaneously introduced into the cell medium. Various GSK-3β inhibitors are known and have been used as a tool in a great number of studies to illuminate the role of this kinase in cellular signaling, physiological events, and different diseases. In this study, two structurally distinct GSK-3β inhibitors were applied. Indirubin-3′-monoxime,5-iodo is characterized by IC$_{50}$ ∼ 10 nM toward GSK-3β; however, like other substituted indirubins it inhibits as well the activity of cyclin-dependent kinases (CDK) [23]. AR-A014418, another specific inhibitor of GSK-3β, is a member of the family of thiazol derivatives and is considered to be the first known compound that does not significantly inhibit the activity of CDK2 or CDK5 [24]. For these reasons, along with indirubin, we applied AR-A014418 as GSK-3β inhibitor in this study. Thus, it could be shown
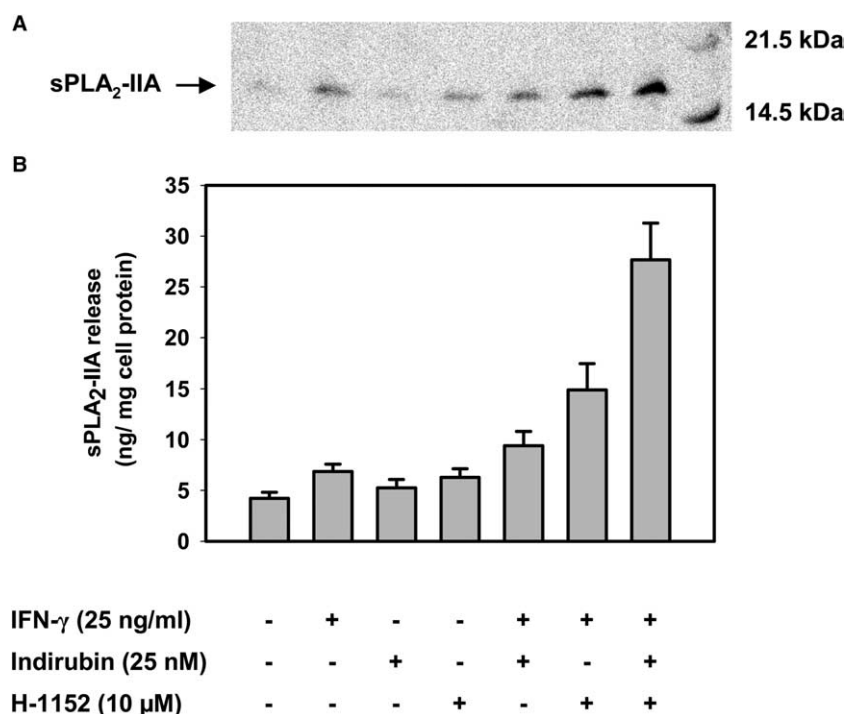
A

sPLA$_2$-IIA →

21.5 kDa

14.5 kDa

B

Fig. 4. Stimulation of sPLA$_2$-IIA secretion after exposure of human HepG2 cells to indirubin alone and in combination with IFN-γ and Rho-kinase inhibitor, H-1152 for 48 h. (A) Immunoblot showing the released sPLA$_2$-IIA protein by using polyclonal anti-sPLA$_2$-IIA antiserum, 30 μg of protein was applied per lane. (B) Quantification of released sPLA$_2$-IIA protein using the ELISA technique. Results are means ± S.D. of analysis in triplicate and are representatives of three independent experiments.

that the results achieved with indirubin were reproducible using AR-A014418.

The proof of a role of GSK-3β in the regulation of sPLA$_2$-IIA is of special interest because several transcription factors important to the regulation of sPLA$_2$-IIA serve as substrates for GSK-3β. Among these, for example, is the transcription factor NF-κB, whose function is strikingly altered by GSK-3β [25–27]. The extent to which GSK-3β activates or blocks NF-κB signaling is the subject of some discussion (for review [28]). On the one hand, studies with mice lacking GSK-3β have suggested that GSK-3β is required for NF-κB activation [29]. On the other hand, several new studies provided evidence for an inverse association between the activity of GSK-3β and NF-κB signaling. For example, Rao et al. [30] have recently shown that in renal medullary interstitial cells the expression of cyclooxygenase-2 (COX-2) is negatively regulated by GSK-3β, in that the inhibition of GSK-3β leads to an activation of COX-2 via induction of NF-κB-dependent pathways. Sanchez et al. [31] described in their study that an overexpression of GSK-3β in astrocytes is associated with the inhibition of NF-κB activation. This was due to a stabilization of the NF-κB-inhibitory protein, IκBα, and a downward regulation of IκB kinase activity. Furthermore, Bournat et al. [32] and Nemeth et al. [33] found that lithium which inhibits GSK-3β has increased the NF-κB activity in the rat pheochromocytoma cell line PC12 and human intestinal epithelial cells, respectively. The reason for apparent discrepancy in modulatory effects of GSK-3β on NF-κB activity remains to be resolved. In the present study, it could be shown that inhibitors of NF-κB (CAPE, PTDC, and PSI) and of Jak2 (AG-490) completely

obviate an increase in sPLA$_2$-IIA synthesis and secretion caused by GSK-3β inhibitors. This leads to the conclusion that both the NF-κB and Jak2/STAT pathways take part in the upward regulation of sPLA$_2$-IIA expression triggered by the inhibitors of GSK-3β.

If the RhoA/Rho-kinase pathway is inhibited as well as that of GSK-3β, this leads to a synergistic sPLA$_2$-IIA increase following the separate inhibiting of both pathways. An explanation for this amplification could be that GSK-3β is activated upstream by the RhoA/Rho-kinase pathway, as described by Sayas et al. [19]. In this connection, it is remarkable that the inhibition caused by Jak2 alone or by NF-κB alone completely hindered the synergistic effect of IFN-γ, GSK-3β, and RhoA/Rho-kinase inhibitors, which leads to the conclusion that for the optimal induction of sPLA$_2$-IIA expression both, e.g., Jak2- and NF-κB-dependent, signaling pathways must be equally active.

The inhibition of GSK-3β activity by low-molecular natural or artificial substances is believed to be variously beneficial, for example, in the treatment of neurodegenerative diseases, diabetes type II, bipolar disorders, stroke, cancer, and chronic inflammatory disease (for review [34]). To the extent that some of these beneficial effects stand in conjunction with sPLA$_2$-IIA induction described here bears further study. It is, however, known that an overexpression of GSK-3β goes hand-in-hand with an amplified apoptosis and, vice versa, that inhibitors provide protection from apoptosis caused by hyperactive GSK-3β [35]. The mechanism behind this is as yet unexplained. In this connection, it is remarkable that anti-apoptotic properties have been ascribed
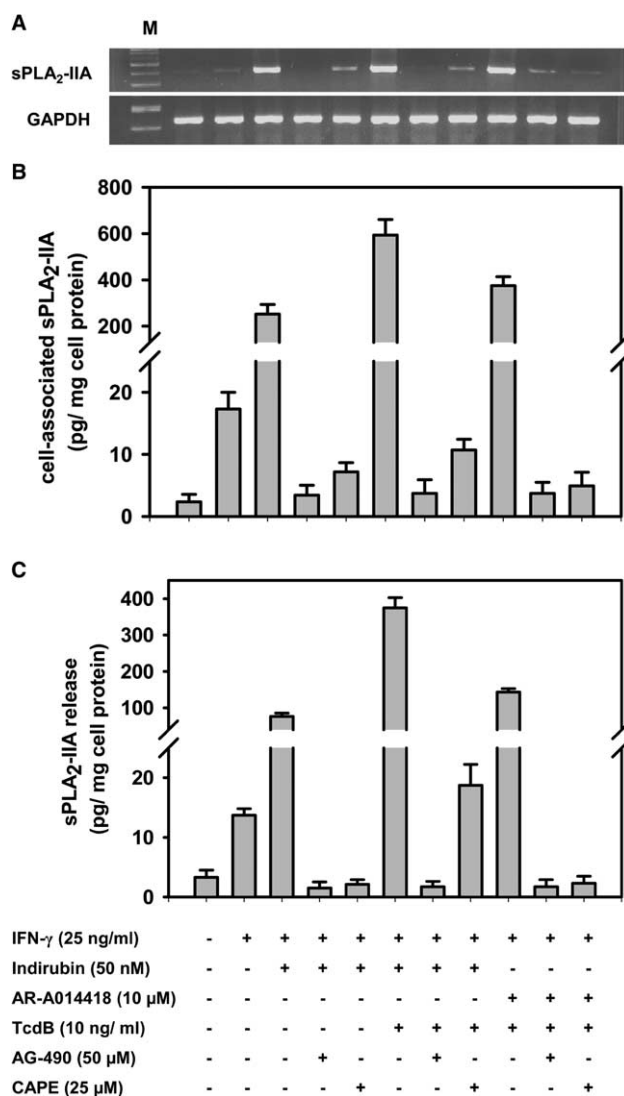
Fig. 5. Effects of Jak2 inhibitor, AG490, and NF-κB inhibitor, CAPE, on the indirubin-, AR-A014418-, and TcdB-mediated upregulation of the sPLA$_2$-IIA in the presence of IFN-γ (25 ng/ml) in HASMC. Cells were incubated for 48 h with indicated additions. (A) sPLA$_2$-IIA mRNA in relation to GAPDH mRNA after RT-PCR in the agarose gel electrophoresis. M indicates 100 bp molecular weight ladder. (B) Quantification of the cell-associated sPLA$_2$-IIA protein. (C) Quantification of the cellular sPLA$_2$-IIA protein released using the ELISA technique, respectively. Results are means ± S.D. of analysis in triplicate and are representatives of three independent experiments.

to sPLA$_2$-IIA [36] and the induction of sPLA$_2$-IIA expression by inhibition of GSK-3β coincides with that, which could be a possible cause of minimized apoptosis.

In conclusion, the study demonstrates that the GSK-3β takes part in a negative regulation of sPLA$_2$-IIA expression. Thus, the inhibition of the GSK-3β alone and especially in combination with the RhoA/Rho-kinase pathway leads to a markedly increased synthesis and secretion of sPLA$_2$-IIA when HASMC and HepG2 cells are simultaneously stimulated with IFN-γ. The mechanism of the negative regulation seems to have as its basis the inhibition of the Jak/STAT- and NF-κB-dependent signaling pathways, in that by means of the inhibition of Jak2 and/or NF-κB the inductive effect of GSK-3β and RhoA/Rho-kinase inhibitors on the IFN-γ-mediated

sPLA$_2$-IIA upregulation is nullified. The physiological relevance of this observation is reserved for further studies, which first will investigate the function of sPLA$_2$-IIA during inflammation.

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