The non-steroidal anti-inflammatory drug aspirin and its metabolite, sodium salicylate, have profound effects on cellular protein synthesis and cell physiology. However, the underlying mechanism by which they cause these responses remains unclear. We show here that salicylates induce phosphorylation of the α-subunit of eukaryotic translation initiation factor 2 (eIF2α), resulting in the inhibition of mRNA translation in cells. Exposure of cells to acetyl salicylic acid resulted in strong activation of eIF2α stress-activated protein kinase R-like endoplasmic reticulum kinase (PERK). Analysis of fibroblasts with a targeted deletion of the perk gene revealed that PERK is indispensable for triggering the phosphorylation of eIF2α as well as the inhibition of protein synthesis induced by salicylates. Although salicylate treatment did not trigger activation of inositol-requiring enzyme 1, there was an increased expression of the pro-apoptotic transcription factor CHOP (gadd153), a downstream event to eIF2α phosphorylation known to mediate endoplasmic reticulum-stress mediated responses. Thus, salicylates selectively trigger an endoplasmic reticulum-stress responsive signaling pathway initiated through activation of PERK to induce their cellular effects.

The non-steroidal anti-inflammatory drugs (NSAIDs) sodium salicylate and aspirin have been widely used to prevent and treat a number of diseases including inflammation and cancer (1,2). Modulation of inflammation by salicylates is, at least in part, due to their ability to inhibit prostaglandin H synthase (cyclooxygenase; COX) activity (3). Aspirin (ASA) efficiently and irreversibly blocks the activity of cyclooxygenases by acetyling specific residues in the COX protein. Salicylic acid (sodium salicylate; NaSal), the aspirin metabolite, is a very poor COX inhibitor, but it is nevertheless able to suppress inflammation at doses comparable to those of ASA (4). The cellular pathways involved in the COX-independent activities of NaSal and other salicylic acid-derived compounds (salicylates) have been at least partially identified (5). For example, it is known that salicylates can both trigger activation of p38 mitogen-activated protein kinase (MAPK) and also inhibit the activation of nuclear factor-κB (NF-κB), thus inducing apoptosis via p38 MAPK and preventing NF-κB-regulated inflammatory gene expression (6-8). It has also been reported that ASA and NaSal act as potent inhibitors of protein synthesis in guinea pig gastric mucosal tissue and rat islets (9,10). However, the molecular mechanism responsible for this effect remains unclear.

The α-subunit of eukaryotic translation initiation factor 2 (eIF2α) is a component of the stable ternary complex formed by Met-tRNA^Met, GTP and eIF2. Formation of this complex is an essential step in the assembly of the 40S ribosomal subunit and subsequent protein synthesis (11). Under a variety of stress conditions, eIF2α is phosphorylated on serine 51 by specific stress-activated kinases. This modification leads to a reduction in the rate of mRNA translation (12). Four mammalian eIF2α kinases have been
identified so far. The general control of amino acid biosynthesis kinase (GCN2) is specifically activated in response to amino acid deprivation (13-16). The hemin-regulated inhibitor kinase was identified in reticulocytes and found to inhibit protein synthesis in heme-deprived lysates and stressed cells (17-19). A third eIF2α kinase, protein kinase R (PKR), is activated by double-stranded RNA of viral or synthetic origin, thereby shutting down cellular mRNA translation and inhibiting virus replication (20). Finally, a PKR-like endoplasmic reticulum (ER) kinase (PERK) has been identified as pancreatic eIF2α kinase (21) and as ER-resident kinase (22). PERK is activated in response to accumulation of misfolded proteins in the ER, reducing the rate of protein synthesis through eIF2α phosphorylation in order to assure proper protein folding (23).

Cellular responses to severe ER perturbations, such as the unfolded protein response, may also include increased expression of ER-localized proteins containing KDEL motifs (24-26). In mammalian cells, transcriptional activation of the pro-apoptotic transcription factor CHOP/gadd153 (27) is observed following ER stress caused by agents such as thapsigargin, tunicamycin, dithiothreitol (DTT) or amino acid starvation. This may occur in a PERK-dependent manner as a consequence of a selective increase in translation of the transcription factor ATF4 or in an ATF3-dependent manner (14,26,28,29). In addition to PERK, another mammalian ER transmembrane protein, mIRE1β, has been shown to regulate CHOP/gadd153 expression (30). This redundancy suggests that cellular responses initiated by ER stress may utilize multiple pathways to regulate translation and subsequent events. Additionally, although the mechanism linking ER stress to caspase activation remains unclear (23), it is possible that ER-mediated stress signals induce programmed cell death via CHOP/gadd153 (27).

Given that exposure of cells to NaSal or ASA results in inhibition of protein synthesis, we looked at the ability of these drugs to elicit phosphorylation of eIF2α and at the activation of its upstream regulators and downstream effectors. Here we show that salicylates inhibit cellular protein synthesis by inducing phosphorylation of eIF2α in a PERK-dependent manner. As a reflection of ER-stress mediated responses, salicylates induced expression of the transcription factor CHOP(gadd153) and caused degradation of caspase-12. These findings characterize a specific signaling pathway, initiated in the ER, that is triggered by salicylates to mediate their anti-proliferative actions.

Pharmacological and suprapharmacological concentrations of salicylates were used in this study. The high concentrations were used as a more effective response was observed. We have shown that salicylate concentrations in the range used therapeutically had the ability to cause a specific cellular response. A large number of studies have reported the use of high concentrations of salicylates, so that the potential effects observed in in vitro studies can be translated to clinical applications. It should be considered that after ingestion of therapeutic doses of salicylates, such as aspirin, some organs (e.g. stomach, kidney) or inflamed areas are exposed to particularly high concentrations of these drugs. The results from our in vitro studies obtained with high concentrations suggest that locally high salicylate concentrations may contribute to trigger a specific ER stress response that could be beneficial in clinical settings such as cancer treatment.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—The human promonocytic cell line THP-1 was grown in 10% FBS RPMI medium supplemented with 2 mM L-glutamine, penicillin and streptomycin, in 5% CO2 at 37°C. SV40-immortalized PERK+/+ and PERK-/- fibroblasts (a kind gift from Heather Harding and David Ron, New York University School of Medicine, New York) were grown in 10% FBS DMEM medium containing penicillin and streptomycin. NaSal and ASA were purchased from Sigma. DTT was purchased from Invitrogen. NaSal was prepared at 1 M concentration in deionized water and then passed through a 0.2 μ filter. ASA was dissolved at 1 M final concentration into dimethylsulfoxide (DMSO) prior to treatments.

Immunoblotting Analysis—Following treatments, cells were washed twice in ice-cold PBS. Cell extracts were obtained with lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM sodium
chloride, 50 mM sodium fluoride, 10 mM β-
glycerophosphate, 0.1 mM EDTA, 10% glycerol, 1 % Triton X-100, 1 mM PMSF, 2 mM sodium
orthovanadate, and 2 μg/ml of pepstatin, leupeptin and aprotinin). Following incubation on ice for 10
min, cell extracts were clarified by centrifugation
at 12,000 rpm for 20 min. Proteins were assayed
for Bradford reagent (BioRad Protein Assay). Cell
extracts were fractionated onto sodium dodecyl
sulfate-polyacrylamide gel electrophoresis (SDS-
PAGE), and transferred to PVDF membranes
(Immobilon-P, Millipore). Anti-caspase-12 and
phospho-site specific antibodies against eIF2α
(Ser51) and PERK (Thr980) were from Cell
Signaling. CHOP(gadd153) antibody was kindly
provided by Dr. Edward Maytin (Dept. of
Biomedical Engineering, Cleveland Clinic
Foundation).

Metabolic Cell Labeling and
Trichloroacetic Acid (TCA) Precipitation—One
day before the experiment, THP-1 cells were
washed twice with PBS and plated at a density of
10⁶ cells/ml in 60-mm dishes. Following treatment
with NSAIDs, cells were harvested by
centrifugation and washed twice in PBS. Cell
labeling in methionine-free medium was followed
by addition of [³⁵S]methionine Revidue (50
μCi/ml; 1,000 Ci/mmol; Amersham) and
incubation for additional 20 min at 37°C. Cells
were washed twice with cold PBS and protein
extracts obtained in lysis buffer (the same as used
for immunoblotting analyses). Thirty-micrograms
of cell extracts were fractionated onto 12% SDS-
PAGE, stained by Coomassie blue, dried and
exposed for autoradiogram analysis. To monitor
the incorporation of [³⁵S]methionine into total
cellular proteins, cellular extracts were assayed for
tCA precipitation and analyzed by scintillation
counting. Briefly, thirty-micrograms of cell
extracts was added to 500 μl of 100 μg/ml bovine
serum albumin containing 0.01% NaN₃. Following
incubation on ice for 5 min, 500 μl of ice-cold
20% TCA solution was added, and incubated for
an additional 30 min on ice. The suspension was
filtered through glass microfiber disks (Whatman
GF/C) and washed twice with 5 ml ice-cold 10%
TCA, and twice with ethanol. Dried-air disks were
transferred to scintillation vials, 5 ml of
scintillation liquid was added and radioactivity
measured by scintillation counting. Results were
normalized as the percent increase or decrease of
[³⁵S]methionine incorporation compared to that of
control untreated cells.

Mammalian Cell Lysate Preparation and
Polyribosome Analysis—THP-1 cells (grown in
suspension at 2 x 10⁶ cells/ml) were treated (prior
to collection) for 10 min with cyclohexemide (CX)
(100 μg/ml final concentration), then washed
twice with PBS buffer (containing 100 μg/ml CX)
and collected by centrifugation (10 min at 3500
rpm). Cell pellets were further resuspended in ice-
cold cell lysis buffer, containing 10 mM HEPES-
KOH pH 7.9, 2.5 mM MgCl₂, 100 mM KCl, 1
mM DTT, 0.1% NP-40, 100 units/ml RNase
inhibitor and 100 μg/ml CX. Cells were
homogenized by passing through a 25 gauge syringe
and the suspension was centrifuged at
6000 rpm for 15 min to remove mitochondria and
cellular debris. Cell extracts (2-5 OD₂₆₀ units)
were loaded on top of linear 7-50% sucrose
gradients in 10 mM HEPES-KOH pH 7.9 buffer,
containing 2.5 mM MgCl₂, 100 mM KCl and 1
mM DTT. Centrifugation was done for 18 h at
17,000 rpm using a Beckman SW32.1 rotor.
Gradients were unloaded by upward displacement
using a programmable density gradient system
with a UV (UA-6) detector (TELEDYNE ISCO,
Inc.). Cell extracts prepared from the control cells
(not treated with ASA or NaSal) or treated with
ASA and NaSal were used.

RT-PCR and Restriction Endonuclease
Analysis of X-Box Binding Protein 1
(XBP-1) mRNA Processing—One-microgram of
total RNA derived from untreated or treated
SV40-immortalized PERK wild-type and knock-
out mouse fibroblasts was reverse-transcribed
using oligo-dT12-18 in the presence of MMLV
reverse-transcriptase SuperScript II (Invitrogen).
One-tenth of the cDNA first-strand reaction was
used as template in PCR to amplify a 600-bp
cDNA segment of XBP-1 mRNA encompassing
the cleavage sites of inositol-requiring enzyme 1
(IRE1) (a small 26-bp intron that contains a
Pst I restriction site within). Primers sequences used in
the PCR assay are the same as described by Calfon
and colleagues (31). One-third of the PCR volume
was then digested with Pst I restriction
endonuclease at 37°C for 150 min to reveal the
restriction site, which is lost following XBP-1
mRNA cleavage by IRE1. PCR and digestion
reactions were fractionated onto 1.2% agarose gel and stained with ethidium bromide.

RESULTS

Inhibition of Protein Synthesis by Salicylates in Human Promonocytic Cells—To determine whether NaSal and ASA affect the rate of protein synthesis in cultured cells, we performed a metabolic cell labeling experiment by measuring $[^{35}\text{S}]$methionine incorporation into nascent proteins. The human promonocytic cell line THP-1 was treated with NaSal (20 mM) or ASA (10 mM) for 1, 3, 6 or 9 h. By comparing total nascent cellular protein labeled with $[^{35}\text{S}]$ (Fig. 1A, upper panels) to total cellular protein as visualized by Coomassie blue stain (Fig. 1A, lower panels), the relative amount of protein synthesis occurring in each sample was determined. Treatment of cells with DTT was used as a positive control and resulted in a dramatic inhibition of protein synthesis. At 1 and 3 h after treatment of the cells with NaSal, there was significant inhibition of protein synthesis followed by a partial recovery at 6 and 9 h. Inhibition of protein synthesis by ASA was also observed, although it was delayed and less pronounced, and appeared at 6-9 h. The extent of protein synthesis inhibition was quantitated by TCA precipitation of the extracts, counting the incorporated $[^{35}\text{S}]$ and compared with the untreated control (Fig. 1C). These results show that there was at least a 40% reduction in $[^{35}\text{S}]$ incorporation into nascent proteins when cells were exposed to NaSal for 1 or 3 h or to ASA for 6 or 9 h. The transient nature of the observed inhibitory effect suggests that inhibition of protein synthesis by salicylates at these concentrations is reversible.

Having determined the optimal time for salicylate treatment that led to the inhibition of protein synthesis, we next investigated the dose-response for these compounds. Inhibition of protein synthesis clearly increased with increasing doses of either NaSal or ASA, with a maximal effect of 98% reduction in $[^{35}\text{S}]$ incorporation into nascent proteins following exposure of the cells to 20 mM ASA for 6 h (Fig. 1B, D). Treatment of cells with an equivalent volume of the ASA solvent, DMSO (lane D), did not significantly attenuate protein synthesis, confirming the specificity of the effect seen with 20 mM ASA.

Although NaSal and ASA affect protein synthesis with distinct kinetics and dose-response curves, there are times and doses at which similar inhibition is noticed with the two drugs (Fig. 1C, D). At higher concentrations, ASA is a much more potent inhibitor of protein synthesis than NaSal.

Exposure of Cells to Salicylates Results in Phosphorylation of eIF2α—Given that phosphorylation of the α subunit of the translation initiation factor eIF2 is known to inhibit mRNA translation, we investigated whether the inhibitory effects of salicylates on protein synthesis were due to salicylate-induced phosphorylation of eIF2α. Protein extracts from THP-1 cells that were treated with increasing concentrations of salicylates were analyzed by Western blot with phospho-specific anti-eIF2α (Ser51) antibody (Fig. 2A). The results show that phosphorylation of eIF2α is induced by treatment of cells with as little as 5 mM NaSal or ASA. At 10 mM, NaSal and ASA induced phosphorylation of eIF2α to a similar extent. A higher dose (20 mM) of NaSal led to more robust eIF2α phosphorylation, but a similar dose of ASA was even more effective at inducing this phosphorylation event. Treatment of cells with an equivalent volume of the ASA solvent DMSO (lane D) as the 20 mM ASA treatment did not result in phosphorylation of eIF2α, confirming the specificity of the ASA effect. As we observed that inhibition of protein synthesis caused by NaSal occurred earlier (at 60 min) and was stronger (38% decrease), we also determined that this effect is directly correlated with eIF2α phosphorylation (Fig. 2B)

Exposure of Cells to Salicylates Results in Inhibition of mRNA Translation—As a result of eIF2α phosphorylation, a dissociation of polyribosomes and accumulation of ribosomal subunits occurs in stressed cells, thus resulting in dramatic inhibition of mRNA translation initiation (32). To provide further direct evidence that ASA and NaSal cause inhibition of translation initiation in vivo, we compared the polysome profiles obtained after fractionation of cell extracts (prepared from untreated and treated cells) through 7-50% sucrose gradients. As shown in Fig. 3 (A, B), both ASA and NaSal led to a substantial decrease in the heavier polysome content and a commensurate increase in 80S monosomes in cells. The reduction in polysome: 80 monosome
ratios for these experiments is characteristic of a reduced rate of translation initiation. We thus further concluded that both compounds impair translation initiation. These results indicate that the inhibition of protein synthesis observed in THP-1 cells (Fig. 1) relies on a mechanism involving components of the translation initiation machinery.

**PERK is Activated by Aspirin Treatment and is Required for Salicylate-Induced Phosphorylation of eIF2α and Inhibition of Protein Synthesis**—Two stress-activated protein kinases, PKR and PERK, are known to mediate stress-induced eIF2α phosphorylation (23,33). However, PKR-mediated eIF2α phosphorylation is largely restricted to virus-infected cells, whereas a number of stress stimuli trigger the PERK-eIF2α module. Therefore, we first tested whether PERK was responsible for salicylate-induced eIF2α phosphorylation. To address this question, the phosphorylation of eIF2α was determined in salicylate-treated PERK-null and wild-type immortalized mouse embryonic fibroblasts. The expected phenotype of these cells was confirmed by noticing induction of eIF2α phosphorylation induced by DTT in wild-type, and not in PERK-null cells (Fig. 4A). While eIF2α phosphorylation increased in the wild-type cells following 5 min of exposure to NaSal, there was no induction of eIF2α phosphorylation in PERK-null cells. However, at 20 min following NaSal treatment, the phosphorylation of eIF2α remains similar in both wild-type and PERK-null cells, decreasing in the latter at 60 min. This transient nature suggests that PERK is the protein kinase required to trigger the initial eIF2α phosphorylation induced by NaSal.

We have shown here that high concentrations of ASA induce robust phosphorylation of eIF2α and dramatically inhibit protein synthesis. To demonstrate that PERK is required for the onset of ASA-induced eIF2α phosphorylation, wild-type and PERK-null fibroblasts were exposed to ASA for 5, 20 or 60 min. The result (Fig. 4B) shows that following 5 and 20 min of treatment, eIF2α phosphorylation is robustly induced in PERK-wild-type, and not in PERK-null cells, suggesting that PERK plays a key role in the phosphorylation of eIF2α induced by ASA. Albeit to a lesser extent than in wild-type cells, following 60 min of ASA treatment, eIF2α phosphorylation could be observed in PERK-null cells.

Having demonstrated that the onset of ASA-induced phosphorylation of eIF2α requires PERK, we next tested whether ASA induces activation of PERK. To this end, we used a phospho-specific anti-PERK antibody that recognizes the activated form of PERK. The result (Fig. 4C) shows that PERK undergoes autophosphorylation at Thr980 following exposure of cells to ASA as with DTT, a known PERK activator (34). Comparison of PERK activation in PERK-null and wild-type cells confirmed both the antibody specificity and the genotype of the cells used in this experiment.

To determine whether there was a PERK-dependent effect on protein synthesis in ASA-treated cells, [35S]methionine incorporation into cellular proteins was determined and analyzed. When wild-type cells were treated with either ASA or DTT (Fig. 5), a dramatic reduction in protein synthesis occurred. In PERK-null cells, however, protein synthesis was not significantly affected by either DTT or ASA. This suggests that, as with DTT, inhibition of protein synthesis triggered by ASA is mediated through activation of PERK. Nevertheless, a residual inhibitory effect of ASA on protein synthesis in PERK-null cells is observed, suggesting a distinct mechanism independent of PERK.

**Salicylates Induce the Expression of ER-Stress Regulated Transcription Factor CHOP(gadd153) and Cleavage of ER-Resident Caspase-12, but do not Trigger the Activation of IRE1**—Phosphorylation of eIF2α and the subsequent inhibition of mRNA translation initiation have been shown to be followed by up-regulation of ER-stress responsive transcripts, including that encoding the transcription factor CHOP(gadd153) (14,35,36). We then determined whether the latter downstream event occurred following salicylate treatment. The results (Fig. 6A, B) show that CHOP(gadd153) has its expression strongly induced in cells following salicylate treatment in cells. Up-regulation of the pro-apoptotic transcription factor CHOP(gadd153) in response to ER-stress has been shown to be PERK dependent (14). Our results show that the expression of CHOP(gadd153) induced by salicylates is partially dependent on PERK. In
contrast, expression of CHOP\textit{(gadd153)} induced by DTT was significantly reduced in PERK-null cells. In addition, we found that while the SV40-immortalized fibroblasts used in these experiments express high levels of the eIF2\textit{α} kinase PKR, this kinase is dispensable for the up-regulation of CHOP\textit{(gadd153)} induced by NaSal (data not shown).

It has been shown that activation of IRE1 is necessary to drive the expression of CHOP\textit{(gadd153)} in response to stress stimuli (30). To determine whether salicylates induce IRE1 activation in cells, we carried out RT-PCR analysis to detect IRE1-mediated splicing of XBP-1 transcription factor (31,37). Our results depicted in Fig. 6\textit{C} indicate that ASA or NaSal (top panel), but not DTT or tunicamycin, failed to activate IRE1 as detected by the XBP-1 mRNA processing. This suggests that CHOP\textit{(gadd153)} expression induced by salicylates most likely is independent of IRE1.

Another hallmark event of ER-stress mediated responses is the proteolytic activation of caspase-12 (38). Caspase-12 degradation occurs only after prolonged ER stress. To provide further evidence that salicylates affect ER homeostasis, we analyzed caspase-12 expression by Western blot in wild-type and PERK-null cells following treatment with DTT or two distinct concentrations of salicylates. We observed that untreated PERK-null cells had higher levels of caspase-12 protein than untreated wild-type cells (Fig. 7), corroborating the findings of Harding and colleagues (26). However, in both cell types, caspase-12 was degraded following exposure to either DTT or salicylates. Degradation in response to salicylates occurred in a concentration-dependent manner.

DISCUSSION

Inhibition of cellular protein synthesis by salicylates has been reported elsewhere (9,10). However, the molecular mechanism responsible for this effect remained unclear. Our findings provide evidence for a mechanism, initiated in the lumen of the ER, by which salicylates trigger inhibition of protein synthesis. Our results demonstrate that inhibition of protein synthesis by salicylates correlates with phosphorylation of eIF2\textit{α} (Figs. 1, 2A). PKR-like kinase PERK was required to initiate phosphorylation of eIF2\textit{α} and mediate inhibition of protein synthesis in response to salicylates (Figs. 4A, B, 5). We also found that PERK was activated by salicylates (Fig. 4C), and that was required to promote the inhibition of global protein synthesis (Fig. 5). Treatment of cells with salicylates led to up-regulation of CHOP\textit{(gadd153)} and cleavage of caspase-12 (Figs. 6A, B, 7), hallmark events of ER stress (14,27,38,39). These results indicate that a specific signaling pathway involving the ER mediates the effects of ASA and NaSal on cells. However, salicylate-induced expression of CHOP\textit{(gadd153)} was only partially dependent on PERK and did not require PKR either (Fig. 6A, B, and data not shown). Its expression is probably not mediated through IRE1 (Fig. 6C), since salicylates did not induce IRE1-mediated processing of XBP-1 mRNA transcription factor mRNA. These observations suggest that an additional eIF2\textit{α} kinase (14) or parallel ER-stress players might contribute to salicylate-induced CHOP\textit{(gadd153)} activation (40-42). IRE1 acts as activator of gene expression in the ER cellular responses (43), whereas PERK is responsible for translational regulation, even though ER-stress compounds induce CHOP\textit{(gadd153)} expression in a PERK-dependent manner (23). Thus, it is likely that salicylates will affect both the activity of components related to the ER-stress pathway and the activation of additional eIF2\textit{α} kinase. The observations above have led us to propose that salicylates may bind to a cellular component localized in the ER. A member of a family of glucose-regulated stress chaperones, Bip/GRP78, is responsible for maintaining proteins in a folding-competent state in the ER (26). Bertolotti et al. showed that PERK is found in a complex with Bip/GRP78 in cells without ER-stress conditions (34). Notably, these authors demonstrated a time-dependent dissociation of the complex following ER-stress induced by DTT or thapsigargin. Interestingly, a report published a few years ago demonstrated that NaSal and ASA can bind \textit{in vitro} to Bip/GRP78 (44), although these authors did not observe induction of ER-stress markers following NaSal stimulation of human foreskin fibroblasts. Given these findings, it is tempting to speculate that the early events in salicylate-induced mRNA translation inhibition may involve binding of the drug to the Bip/GRP78-PERK complex in the ER,
followed by PERK dissociation, dimerization, auto-activation and substrate (eIF2α) phosphorylation.

The data presented here do not exclude the possibility that additional components activated from ER stress might be involved. Indeed, there are other ways in which salicylates might impinge on ER physiology. For example, salicylates can uncouple oxidative phosphorylation and thus affect ATP production (45), and at high doses they may also affect glucose transport into cells (46). Both stresses activate the unfolded protein response, which takes place in the ER. Nonetheless, further experiments will need to be carried out to better clarify this issue.

Pharmacological and supra-pharmacological doses of salicylates have been used in this study so that we could elucidate the mechanism by which these pharmacological compounds affect protein synthesis and the activity of components in the ER. It must be mentioned that the phosphorylation of eIF2α and the inhibition of protein synthesis are noticed at concentrations that approach those used in specific therapeutic settings, i.e. in the range of 1-5 mM (47). In addition to these observations, it must also be considered that high concentrations of ASA and NaSal are found in inflamed tissues and some organs, which may account for some of their clinical and side effects (48). Our results justify further investigation into the effects of salicylates on translational machinery and ER homeostasis in cancer cells (49). Furthermore, our identification of eIF2α phosphorylation and CHOP(gadd153) gene activation as targets of salicylate action may provide insights into the design of aspirin-like drugs with more effective anti-inflammatory or anti-proliferative properties.

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REFERENCES

FOOTNOTES

2The abbreviations used are: NSAIDs, non-steroidal anti-inflammatory drugs; COX, cyclooxygenase; ASA, aspirin; NaSal, sodium salicylate; MAPK, mitogen-activated protein kinase; NFκB, nuclear factor-kB; eIF2α, α subunit of eukaryotic translation initiation factor 2; GCN2, general control of amino acid biosynthesis kinase; PKR, protein kinase R; ER, endoplasmic reticulum; PERK, PKR-like ER kinase; DTT, dithiothreitol; DMSO, dimethylsulfoxide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; CX, cyclohexemide; XPB-1, X-box binding protein 1; IRE1, inositol-requiring enzyme 1.

FIGURE LEGENDS

Fig. 1. Salicylates inhibit protein synthesis in human promonocytic cells. Following treatments, cells were harvested by centrifugation, washed in PBS and nascent proteins were labeled with [35S]methionine (50 μCi/ml) in methionine-free medium. Cells were then washed twice in cold PBS and cell lysates were obtained. Equal protein loading was fractionated onto 12% SDS-PAGE, Coomassie blue stained (lower panels), dried and exposed for autoradiogram analysis (upper panels). A, Autoradiogram of 35S-labeled proteins in SDS-PAGE showing a time dependency of protein synthesis inhibition by NaSal and ASA. A day before treatment, THP-1 cells were plated in 60-mm dishes (1 x 10⁶ cells/ml). Cells were treated with NaSal (20 mM) or ASA (10 mM) for the time indicated on the figure. Cells were also exposed to DTT (10 mM). B, Autoradiogram of 35S-labeled proteins in SDS-PAGE revealing dose dependency of the inhibition of protein synthesis by NaSal and ASA. As above, 1 x 10⁶ cells/ml were used for the experiment. Cells were treated with NaSal for 3 h or ASA for 6 h with the indicated concentration. Cells were also exposed to ASA-solvent DMSO (lane D) for 6 h. C and D, Analysis of [35S]methionine incorporation into nascent proteins shown in A and B, respectively. Cell extracts were precipitated with TCA and cpm were normalized as described in Experimental Procedures.

Fig. 2. Salicylates induce phosphorylation on Ser51 of eIF2α in THP-1 cells. Following treatments, cells were harvested by centrifugation and washed in PBS. Cell extracts were obtained in lysis buffer, fractionated onto 10% SDS-PAGE and immunoprobbed against phospho-specific eIF2α (p-eIF2αSer51) antibody (1:1000) and non-phosphorylated eIF2α (1:2000). A, Western-blot analysis of dose-response of eIF2α phosphorylation induced by salicylate treatment of cells. Cells (1 x 10⁶ cells/ml) were treated with NaSal (3 h) or ASA (6 h) with the drug concentration indicated. B, Western-blot analysis of eIF2α phosphorylation following NaSal and ASA treatment of cells at early time points. Cells were treated with 20 mM NaSal or 10 mM ASA for the time shown on the figure. Cell extracts were analyzed as above.

Fig. 3. Translation initiation is impaired in ASA- and NaSal-treated cells. Following treatments, cellular extracts were prepared and the fractionation was carried out as indicated in Experimental Procedures. A and B, Polyosomal profile analysis in human THP-1 and mouse embryonic fibroblasts, respectively. Top panels: untreated cells; middle panels: cells treated with 10 mM ASA for 30 min; bottom panels: cells treated with 20 mM NaSal for 30 min. Cellular extracts were fractionated through 7-50% linear sucrose gradients. A continuous record of the absorbance at 260 nm for each gradient is presented, with the top of the gradient on the left. The arrows indicate the peaks for the 40S and 60S subunits, and 80S monoribosomes. The ratio of the area under the polysomal (P) and 80S peaks is shown (P:80S). The data recorded by the PeakTrak program (ISCO gradient Density Gradient Fractionation System) were exported to ASCII format and analyzed by UVProbe 2.10 Shimadzu software to assess the 80S and polysomal peak area. The results are representative of at least two independent experiments.
PERK is activated by salicylates and is required for the onset of eIF2α phosphorylation. SV40 immortalized wild-type or PERK-deficient mouse fibroblasts were left untreated or treated with NaSal, ASA or DTT for various times as indicated in the panels. Cell extracts were obtained, fractionated onto SDS-PAGE, transferred to PVDF membranes and immunoblotted with specific antibodies. A and B, Phospho-eIF2α immunoblots showing that salicylates fail to induce efficient eIF2α phosphorylation in PERK-null cells. Wild-type (+/+) or PERK-null (-/-) mouse fibroblasts were left untreated (time 0) or then treated with NaSal (20 mM) or ASA (20 mM) for the time indicated. The intensity of p-eIF2α and eIF2α necessary to obtain the ratio peIF2α:eIF2α was determined through ImageQuant 5.2 software (Molecular Dynamics) C, Phospho-PERK Western-blot analysis of ASA-induced phosphorylation at the Thr980 residue of PERK. Wild-type (+/+ or PERK-null (-/-) fibroblasts were left untreated or then treated with ASA (20 mM) for the time indicated. Cell extracts were obtained, fractionated onto 8% SDS-PAGE, transferred to PVDF membranes and immunoprobed as shown.

Aspirin inhibits protein synthesis in a PERK-dependent manner. Autoradiogram of 35S-labeled proteins in SDS-PAGE comparing the rate of protein synthesis in wild-type or PERK-null cells exposed to ER-stress agent DTT (10 mM) or ASA (20 mM). Cells were pulse labeled for 10 min with [35S]methionine after 5 or 20 min of treatment with ASA or DTT, and then cell extracts were obtained. Forty-micrograms of total protein was fractionated onto 12% SDS-PAGE, stained with Coomassie blue (right), dried and exposed to X-ray film for autoradiogram analysis (left). U, untreated. The analysis of the [35S]methionine incorporation into nascent proteins is shown at the bottom of the autoradiogram. Cell extracts were precipitated with TCA and c.p.m. were normalized as described in Experimental Procedures.

Salicylates induce the expression of ER-stress regulated gene CHOP(gadd153), but fail to trigger the activation of IRE1. A and B, Western blots showing that PERK is not essential for salicylate-induced expression of CHOP(gadd153). Wild-type or homozygous null PERK fibroblasts were left untreated or treated with NaSal (20 mM), ASA (10 mM) or DTT (10 mM) for the times indicated. Cell extracts were fractionated onto SDS-PAGE, transferred to PVDF membranes and immunoprobed as indicated. The relative levels of CHOP expression were determined by quantitation of the Western blot bands using densitometry and were normalized against the level of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). The fold increase in CHOP levels for each sample indicated below the autoradiograms is relative to untreated control cells. The result is representative of three independent experiments. C, Captured image of ethidium bromide-stained agarose gel showing of XBP-1 amplicons revealing that salicylates fail to trigger IRE1-mediated cleavage of XBP-1 mRNA in cells. Mouse embryonic fibroblasts were left untreated (UT) or treated with ER-stress inducers DTT (2 mM) and tunicamycin (5 μg/ml), or with salicylates NaSal (20 mM) and ASA (10 mM) for the times indicated. XBP-1 PCR product was generated from the first-strand cDNA synthesized from the total RNA obtained in each treatment indicated on the figure. Non-digested (-) and Pst I-digested (+) PCR products were fractionated onto 1.2% agarose gel. The sizes of unprocessed (600 bp) and processed (576 bp) cDNA fragments are indicated on the figure.

Salicylates induce caspase-12 degradation. Immunoblotting analysis of caspase-12 degradation and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) in mouse fibroblasts. Wild-type (PERK +/+ or PERK-null (PERK-/-) mouse fibroblasts were left untreated or treated for 24 h with NaSal, ASA or DTT with the concentrations indicated on the figure. Cells extracts were fractionated onto SDS-PAGE, transferred to PVDF membranes and immunoprobed with anti-caspase-12 antibody.
Figure 1

A

NaSal 20 mM  DTT  ASA 10 mM

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B

NaSal  ASA  D

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C

35S incorporation (%)

0  1  3  6  9

Time (h)

NaSal  ASA

D

35S incorporation (%)

0  1  5  10  20

Concentration (mM)

NaSal  ASA
Figure 3

A. THP-1

B. Mouse fibroblasts

Top 7%  Bottom 50%

A260

P:80S=3:1

80S 40S 60S

Polyribosomes

Control: untreated cells

A260

P:80S=1:1

80S 40S 60S

Polyribosomes

Aspirin, 10 mM

A260

P:80S=1:3.2

80S 60S 40S

Polyribosomes

Sodium salicylate, 20 mM

A260

P:80S=2.7:1

80S 60S

Polyribosomes

Control: untreated cells

A260

P:80S=1:2.8

80S 60S 40S

Polyribosomes

Aspirin, 10 mM

A260

P:80S=1:3.7

80S 60S 40S

Polyribosomes

Sodium salicylate, 20 mM
## Figure 4

### A

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**Ratio**  
\[
p-eIF2\alpha_{Ser51} : eIF2\alpha
\]

### B

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**Ratio**  
\[
p-eIF2\alpha_{Ser51} : eIF2\alpha
\]

### C

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**Ratio**  
\[
p-\text{PERK}_{Thr980}
\]
**Figure 5**

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**time (min)**

![Coomassie-blue](image)

**35S incorporation (%)**
### Figure 6

#### A

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*Fold induction*

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*Fold induction*

#### C

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#### DTT

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*Fold induction*
Figure 7

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Caspase-12

GAPDH
Salicylates trigger protein synthesis inhibition in a PKR-like endoplasmic reticulum kinase-dependent manner
Aristobolo M. Silva, Die Wang, Anton A. Komar, Beatriz A. Castilho and Bryan R. G. Williams

J. Biol. Chem. published online February 6, 2007

Access the most updated version of this article at doi: 10.1074/jbc.M609996200

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