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Aspirin may promote mitochondrial biogenesis via the production of hydrogen peroxide and the induction of Sirtuin1/PGC-1 α genes

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Abstract

Based on the rapid hydrolysis of acetyl salicylic acid (ASA, Aspirin) to salicylic acid (SA), the ability of SA to form dihydroxy benzoic acid (DBA), and the latter's redox reactions to yield hydrogen peroxide (H_2O_2), we predicted that ASA may have the potential to induce Sirtuin1 (Sirt1) and its downstream effects. We observed that treatment of cultured liver cells with ASA resulted in the induction of Sirt1, peroxisome proliferator-activated receptor-gamma co-activator-1 α (PGC-1 α), and NAD(P)H quinone oxidoreductase 1 (Nqo1) genes. Paraoxonase 1 (PON1) and Aryl hydrocarbon receptor (AhR) siRNA transfections inhibited the induction of gene expressions by ASA suggesting the need for the acetyl ester hydrolysis and hydroxylation to DHBA. The latter also induced Sirt1, confirming the proposed pathway. As predicted, ASA and SA treatment resulted in the production of H_2O_2 , a known inducer of Sirt1 and confirmed in the current studies. More importantly, ASA treatment resulted in an increase in mitochondria as seen by tracking dyes. We suggest that DHBA, generated from ASA, via its oxidation/reduction reactions mediated by Nqo1 might be involved in the production of O_2^- and H_2O_2 . As Sirt1 and PGC-1 α profoundly affect mitochondrial metabolism and energy utilization, ASA may have therapeutic potential beyond its ability to inhibit cyclooxygenases.

Keywords

Aryl hydrocarbon receptor; Dihydroxy benzoic acid; Mitochondria; Paraoxonase

1. Introduction

Acetyl salicylic acid (ASA) is widely used for the prevention of thrombosis and as analgesic agent. Small dose of ASA is generally prescribed as a deterrent of cardiovascular disease even in asymptomatic subjects (Hennekens, 1998). ASA's action might be primarily on the inhibition of cyclooxygenases (COX) by acetylating the enzyme. It may also beneficially affect other chronic diseases by inhibiting Cox. However, ASA has a short plasma half-life of less than 15 min and is rapidly metabolized to salicylic acid (SA) (Santanam and Parthasarathy, 2006; Vane, 1971; Wu, 2003). SA builds up to very high concentrations depending on the dose of ASA. SA itself has analgesic properties (Thompkins and Lee, 1975).

We and others have reported that ASA and SA induced the expression of aryl hydrocarbon receptor (AhR), an aromatic hydroxylase, which suggested that the generation of, 2, 3- and

2, 5- dihydroxybenzoic acid (DHBA) by this P450 enzyme might play a role in its metabolism (Jaichander et al., 2008; Ingelman-Sundberg et al., 1991). SA has also been extensively used as a hydroxyl radical trap to form 2, 3- DHBA (Grootveld and Halliwell, 1986). As dihydroxy phenols undergo oxidation/reduction reactions and generate O_2^- and H_2O_2 (Philipp et al., 2006) and as H_2O_2 is a known inducer of sirtuin 1 (Sirt1) (Kwon and Ott, 2008; Lavu et al., 2008; Alcendor et al., 2004), we tested the ability of ASA and SA to generate H_2O_2 and to induce Sirt1 and one of its downstream effector, PGC-1 α , known to be involved in the biogenesis of mitochondria (Meirhaeghe et al., 2003; Lavu et al., 2008). We also tested the ability of ASA to increase the number of mitochondria.

2. Materials and methods

2.1. Reagents

ASA, SA, DHBA, NADH, 2, 6-dichlorophenolindophenol (DCPIP), 5- and 6- carboxy-2-7-dichlorofluorescein diacetate bis (aceto-methyl) ester (DCDHF-DA), and 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole (SB203580), an inhibitor of MAP kinase homologs p38 α , p38 β and p38 δ , were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO). Rabbit polyclonal anti-SIRT1 (H-300) and anti-PGC-1 α antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and HRP-conjugated secondary antibodies were purchased from Millipore (Temecula, CA). Immuno-star HRP substrate kit was purchased from Bio-Rad (Hercules, CA). HepG2 cells were purchased from the American type culture collection (Manassas, VA). PCR primers & MitoTracker green dye used for studies were from Invitrogen (Grand Island, NY). NF κ B and MAP kinase signaling pathway PCR array kits were purchased from SABiosciences, (Valencia, CA).

2.2. Cell culture and treatment

HepG2 cells or mouse primary hepatocytes from normal C57BL6 mice were cultured in advanced DMEM and were pre-treated with phenol-red free MEM containing ASA or SA at concentrations between 50 μ M and 250 μ M for a period of 48 h. As published earlier (Jaichander et al., 2008), no cellular toxicity was noted under these conditions.

2.3. Sirt1, PGC-1 α and Nqo1 gene expression

Total RNA from cells, after treatment with ASA or other compounds, was extracted using Trizol (Grand Island, NY) according to manufacturer's protocol. First strand cDNA synthesis was performed from 1 μ g total RNA. Reverse transcriptase products were subjected to PCR amplification with Ready Mix PCR Master Mix (Invitrogen, Grand Island, NY). Oligonucleotide primers were designed on the basis of the cDNA sequences reported in the Gene Bank database. RT-PCR was performed for analyzing Sirt1, PGC-1 α and Nqo1 gene expression.

2.4. Western blot analysis for Sirt1 and PGC-1 α protein in HepG2 cells

Protein was measured by Lowry method using BSA as standard (Lowry et al., 1951). For Sirt1 expression studies, protein was separated on 12% SDS-PAGE gel and transferred to nitrocellulose membrane. After transfer, the membrane was blocked with 5% nonfat dry milk in Tris buffered saline (TBST) containing 0.2% Tween20 for 2 h and probed with primary antibody (1:1000 dilution) in 1x PBS, pH 7.4 containing 1% BSA for overnight at 4 $^{\circ}$ C. Bound antibodies were detected by using HRP-linked secondary antibodies (1:2000 dilution) prepared in TBST. PGC-1 α protein expression was also detected similarly. Sirt1 and PGC-1 α proteins were visualized using enhanced Western Blotting Luminal Reagent from Bio-Rad (Hercules, CA) and autoradiographed.

2.5. NFkB and MAP kinase signaling in mouse primary hepatocytes treated with ASA

NFkB and MAP kinase studies were performed after treatment of hepatocytes with 50 μ M ASA for 48 h. Pathway profiling was done by using RT Profiler PCR Array kit as suggested by the manufacturer. In order to establish the importance of MAP kinase signaling in the proposed mechanism for AhR and Sirt1 induction, mouse primary hepatocytes were treated with SB203580 inhibitor and 50 μ M ASA for 48 h. Hepatocytes were also treated with 50 μ M ASA alone. At the end of treatment, hepatocytes were washed gently with PBS and harvested in Trizol for RNA extraction. AhR and Sirt1 gene expression was studied by RT-PCR for all samples.

2.6. Determination of NAD(P)H quinone oxidoreductase 1 (Nqo1) and Sirt1 activities

Nqo1 activity assay was performed using the following conditions: 200 μ M NADH and 40 μ M DCPIP in 25 mM Tris-HCL containing 0.7 mg/ml BSA. Total reaction mixture for the assay was maintained till 1 ml. After the addition of cytosol, the decrease in absorbance at 600 nm was monitored over 1 min at 27 °C (Benson et al., 1980). Sirt1 activity was determined by using N-acetyl Le-Lysine as substrate (Smith et al., 2009).

2.7. Small interfering RNAs (siRNA) transfection

HepG2 cells were grown in 6-well plates. Cells were transfected using Oligofectamine reagent to give a final concentration of 140 nM Oligofectamine (Invitrogen, Grand Island, NY) containing PON1- targeted siRNA or AhR siRNA and control (nonsilencing) siRNA. Forty-eight hours after transfection of both sets of PON1 siRNA/control RNA and AhR siRNA/control RNA, cells were washed and incubated with fresh medium. Cells were then treated with or without ASA. Real-time PCR was performed by using the iCycler from BioRad, Hercules, CA. Fold induction was calculated according to established formula using reference gene and target gene changes. GAPDH was used as the endogenous control gene. Experiments were run thrice independently.

2.8. Differential confocal microscopy

HepG2 cells were treated with 50 μ M and 0.1 mM ASA, incubated at 37 °C for 48 h. At the end of the treatment, cells were gently washed with PBS and the accumulation of H₂O₂ was determined by using 6-carboxy-2, 7-dichlorodihydrofluorescein diacetate bis (acetomethyl) ester (DCDHF-DA) (Ohba et al., 1994). Similarly, O₂⁻ generation was determined by using dihydroethidium (DHE) by confocal microscopy (Zhao et al., 2005). Fluorescence was visualized on a Leica TCS SP2 confocal microscope (excitation, 510 nm; emission, 590 nm).

HepG2 cells were transfected with AhR siRNA for 48 h after which the cells were washed gently by PBS and treated with 0.1 mM ASA for 48 h. Cells were rinsed gently with PBS, incubated for 30 min with 5 μ mol/L DCDHF-DA at 37 °C and then rinsed 3 times gently with PBS. Fluorescence was visualized on a Leica TCS SP2 Confocal microscope (excitation, 488 nm; emission, 525 nm).

2.9. Staining of HepG2 cells with MitoTracker Green and DAPI

To stain the mitochondria, HepG2 cells were plated on cover-slips. They were pre-incubated in serum-free and phenol-free MEM for 4 h. Cells were then treated with ASA and SA at concentrations of 50 μ M, 100 μ M and 250 μ M. At the end of incubation, cells were washed with HBSS and stained with 20 nM MitoTracker Green (Invitrogen) as per manufacturer's instructions. Nuclei were counterstained with 300 nM DAPI (Invitrogen) in PBS. Cells were observed at 100X oil immersion using Zeiss fluorescence microscope.

2.10. Statistical analysis

All experiments were performed at least 3 separate times with triplicate measurements. The data are expressed as mean $\pm$ S.D. and student t-test was applied for significance at $P<0.05$.

3. Results

3.1. Induction of Sirt1 in HepG2 cells and primary hepatocyte

Sirt1 acts as central regulator of all transcription factors that include PGC-1 α which increases in response to metabolic stimuli and is also known to have a role in mitochondrial biogenesis (Kwon and Ott, 2008; Lavu et al., 2008). Treatment of HepG2 cells with ASA resulted in an induction of Sirt1 at 50 μ M levels. A 3.848 ± 0.01 fold induction in Sirt1 gene expression was noted as compared to control incubations (Fig. 1A). Mouse primary hepatocytes showed significant induction at 50 and 100 μ M ASA (5.216 ± 1.9 and 6.845 ± 3.7 fold respectively) as seen in Fig. 1B. Sirt1 activity as measured by deacetylation of N-acetyl Le-Lysine was also increased in a concentration-dependent manner. Sirt1 activity at 50 and 100 μ M ASA showed an increase of 8.76 ± 0.004 and 25 ± 0.003 , respectively, as compared to control.

3.2. Induction of PGC-1 α in HepG2 and primary hepatocytes

Sirt1 induces PGC-1 α , a regulator of energy metabolic pathways (Kwon and Ott, 2008). Our current studies confirm Sirt1's role in activating this protein. HepG2 cells treated with 50 μ M and 0.25 mM ASA showed increase of 1.99 ± 0.7 and 8.229 ± 1.3 fold induction of PGC-1 α gene, respectively (Fig. 1C). In primary hepatocytes PGC-1 α gene expression (Fig. 1D) showed a significant increase at 50 μ M (85.559 ± 12 fold) and 0.25 mM (231.31 ± 10 fold) ASA treatment as compared to control. Primary hepatocytes are more permeable than cultured cell lines and such differences could contribute significantly to ASA's entry and intracellular metabolism. It is also likely that ASA is better hydrolyzed by primary hepatocytes to SA as compared to cultured HepG2 cell line. Sirt1 and PGC-1 α protein expressions as determined by Western blot analysis also showed an increase after ASA treatment (Fig. 1E). Sirt1 has been reported to be induced by H₂O₂ (Zhu et al., in press). Fig. 1F shows the induction of Sirt1 gene expression in HepG2 cells treated with 50 μ M H₂O₂. A 3.848 ± 0.01 fold induction was noted as compared to controls.

3.3. Induction of Nqo1 gene expression and activity in HepG2 and primary hepatocytes

Based on the induction of AhR (Jaichander et al., 2008) (which hydroxylates SA) we predicted that DHBA might mediate the actions of ASA/SA and induce Sirt1. Accordingly, HepG2 cells treated with DHBA (50 μ M and 0.1 mM) showed an increase in Sirt1 gene expression (Fig. 2A). Dihydroxy aromatic compounds undergo oxido-reduction reactions catalyzed by Nqo1 and generate superoxide radicals and H₂O₂. In HepG2 cells Nqo1 gene expression was increased to 4.81 ± 0.3 (50 μ M ASA) as compared to control shown in Fig. 2B. There was also an increase in Nqo1 activity in 50 μ M ASA treated HepG2 cells as compared to control. The average values are 754.74, 765.06 nmole of DCPIP reduced/min/mg protein in treated duplicate samples as compared to control samples of 346 nmole (data not shown).

3.4. siRNA transfection studies on ASA treated HepG2 cells

In order to demonstrate the importance of PON1 and AhR in ASA metabolism we have performed experiments by silencing both genes in the following experiments. Fig. 2C shows a decrease in Sirt1 gene expression in PON1 siRNA transfected and ASA treated HepG2 cells as compared to 500 μ M ASA treated cells. We chose a higher concentration of ASA to show that even under maximum induction conditions, the siRNA transfections could inhibit

such inductions. We also find similar reduction in Sirt1 gene expression when lower concentrations of ASA were used (data not shown). Additionally, AhR siRNA transfection studies also showed decrease in Sirt1 gene expression after ASA treatment compared to 500 μ M ASA treated cells only (Fig. 2D). These results suggest that both PON1 and AhR might be involved in the actions of ASA.

3.5. Inhibition of AhR and Sirt1 by SB203580 in ASA treated hepatocytes

A role for MAP kinase signaling pathway in inducing AhR and Sirt1 gene by ASA treatment was suggested by the ability of a MAP kinase inhibitor SB203580 to inhibit the induction. Hepatocytes treated with SB203580, a MAP kinase inhibitor, and ASA showed a decrease in AhR and Sirt1 gene expression as compared to ASA treatment alone. A noticeable decrease was observed in AhR gene expression at both 50 μ M aspirin+inhibitor (1.143 ± 0.3), and 0.1 mM aspirin+inhibitor (1.205 ± 0.105) as compared to ASA alone (Fig. 2E). Sirt1 expression was reduced to 1.603 ± 0.01 compared to ASA treated hepatocytes that showed a 9.303 ± 0.02 fold induction (Fig. 2F). We also determined MAP kinase signaling pathway by using a commercial kit (SABio-sciences). MAP kinase dependent genes such as Ccnd3 (Cyclin D3), Egfr, Mapk14 (p38 MAPK), Hprt1 were up regulated, whereas Cdc42, Cdkn1b, E2f1, Egfr1, Elk1, Hspa5, Kcnn1, Kras, Map2k4, Map2k6, Map3k2, Map4k1, Mapk10, Mapk8, Mapk8ip1, Map8ip3, Mapkapk5, Rb1, Smad4 were down regulated when hepatocytes were treated with 50 μ M ASA (data not shown). In contrast to MAP kinase signaling pathway we noticed that NFkB signaling was down regulated (data not shown). The ability of ASA to affect liver specific NFkB signaling has been documented before.

3.6. Confocal microscopic studies for detection of intracellular H₂O₂

We determined the generation of O₂⁻ in ASA treated cells using dihydroethidium (DHE) staining. HepG2 cells treated with 50 μ M, 0.1 mM ASA showed presence of oxyethidium formation that reflected intracellular O₂⁻ production (Fig. 3A).

O₂⁻ spontaneously and enzymatically dismutates into H₂O₂. Therefore, we measured the intracellular oxidized state of DCDHF-DA in ASA treated cells as a measure of the formation of H₂O₂. As shown in Fig. 3B, ASA treated cells showed enhanced fluorescence as compared to control cells. Cells transfected with AhR siRNA showed almost a complete absence of oxidized fluorescent compound 2, 7-dichlorofluorescein. The experiments revealed importance of AhR in the formation of oxygen radicals and H₂O₂ from ASA.

3.7. ASA treatment increases the number of mitochondria in HepG2 cells

In order to test whether the activation of Sirt1/ PGC-1 α actually results in mitochondriogenesis, we tested the ability of ASA and SA to increase the number of mitochondria. HepG2 cells incubated with ASA showed a dose dependent increase in mitochondria as observed by staining with MitoTracker Green dye (Fig. 4A). Cells treated with sodium salicylate also showed an increase in Mitotracker staining after 48 h with increasing concentrations of SA (Fig. 4B).

4. Discussion

Our previous studies showed significant increase in PON1 activity in the medium of liver cells treated with ASA, together with an induction of PON 1 and apo A1 gene expressions. Furthermore, the studies pointed out the importance of AhR in the induction of PON1 and apo A1 gene expression (Jaichander et al., 2008). AhR has been well recognized as an important receptor in the metabolism of many drugs and is primarily involved in hydroxylation reactions. SA, derived by the action of PON1 on ASA, not only has been reported to undergo enzymatic hydroxylation but also has been a target for hydroxyl

radicals, generating isomeric dihydroxy phenols (Ingelman et al., 1991; Grootveld and Halliwell, 1986). Similarly, resveratrol has been known to be metabolized by cytochrome mediated reactions to yield the 2, 3-dihydroxy compound piceatannol (Potter et al., 2002). Dihydroxy aromatic compounds undergo quinone/hydroquinone reactions readily both enzymatically and non-enzymatically via the quinone reductase (Benson et al., 1980; Markus and Morris, 2008). O_2^- and H_2O_2 are generated as a result (Markus and Morris, 2008) (Scheme 1). Our hypothesis based on the involvement of AhR, the quinone reductase pathway, and the ability of H_2O_2 to induce Sirt1 predicted that the latter would be an intermediate in the activation of Sirt1. We therefore measured intracellular generation of O_2^- and H_2O_2 .

Sirt1 has been suggested to be induced by H_2O_2 and our present results corroborate the literature findings (Kwon and Ott, 2008; Lavu et al., 2008; Alcendor et al., 2004). However, whether the production of H_2O_2 could mediate the observed effects of ASA was an intriguing possibility. The production of H_2O_2 appears to be linked to the formation of dihydroxy aromatic compounds and their subsequent metabolism that generates superoxide radicals. It remains to be tested whether other dihydroxy compounds would share similar fate and would be able to induce Sirt1. The results presented in the study clearly shows that ASA-treated cells generate more H_2O_2 and its production depends on the hydroxylation reactions mediated by AhR. Thus, the actions of ASA/SA and resveratrol are probably mediated by their abilities to undergo hydroxylation and oxido-reduction reactions and to generate H_2O_2 .

Sirt1 has been associated with mitochondrial function, thermogenesis, and ageing (Alcendor et al., 2007). Both ASA and resveratrol have been shown to increase the longevity of laboratory mice (Philipp et al., 2006; Nemoto et al., 2005). In this study, the results demonstrate the induction of PGC-1 α , a key factor in the biogenesis of the mitochondria (Nemoto et al., 2005). ASA has been suggested to extend the life span in mice (McIlhatton, 2011). It should be remembered that ASA/SA is mostly metabolized by glucuronylation and only a fraction might undergo hydroxylation reactions. This could suggest that the reaction may not produce the large concentrations of H_2O_2 that could be deleterious to cells.

Studies reported so far state that moderate induction in Sirt1 expression and activity could have significant effect in mitochondrial biogenesis which could be beneficial in cardiomyopathy, diabetics, and aging (Lavu et al., 2008; Alcendor et al., 2004). Our studies provide direct evidence for the increased mitochondria in ASA and SA treated cells. This cannot be directly attributed to H_2O_2 but perhaps due to the defense mechanisms (e.g. induction of catalase) in such cells, as catalase is associated with the mitochondria and has been reported to increase longevity. The ability of oxidants, for example peroxidized lipids, to induce anti-oxidant mechanisms has been well described in literature (Meilhac et al., 2001). Sirt1 is also implicated in adaptive thermogenesis, fatty acid oxidation and cardiac development. Therefore, induction of Sirt1 predicts its beneficial role by preventing cardiac hypertrophy and various other metabolic disorders. Furthermore, modest upregulation of Sirt1 could likely be a compensatory mechanism that retard aging and inhibit apoptosis without causing mitochondrial dysfunction or NAD^+ depletion (Alcendor et al., 2007; North et al., 2003; Pillai et al., 2005). Studies suggest that low to moderate levels (2.5–7.5 fold) of Sirt1 could prevent cardiac hypertrophy, inhibit apoptosis and (or) fibrosis and expression of senescence markers (Alcendor et al., 2007; Nemoto et al., 2005). PGC-1 α increase during metabolic state has been reported to be increased by Sirt1 activation. Sirt1 affects hepatic lipid and mitochondrial metabolism through PPAR, estrogen-related receptor (ERR) and hepatocyte nuclear factor 4 (HNF-4) (Alcendor et al., 2007; Nemoto et al., 2005).

The current results suggest that ASA at therapeutic dose levels could induce Sirt1 and PGC-1 α , which might have beneficial impact in cardiac disease and might play a significant role in various pathological conditions. Paradoxically, a high level of Sirt1 induces mitochondrial dysfunction, which may in turn cause increase in oxidative stress (Alcendor et al., 2007). A deleterious effect has also been emphasized stating that high levels of Sirt1 could consume NAD⁺, thereby causing depletion of NAD⁺. Because NAD⁺ is required for mitochondrial respiration, depletion of NAD⁺ could lead to deficiency in ATP and, consequently, cellular dysfunction and cell death (Alcendor et al., 2007; Clark et al., 2000; Radons et al., 1994; Rodgers et al., 2005; Sala et al., 2003; Slebos et al., 2003; St-Pierre et al., 2006; Pfluger et al., 2008). Thus, it can be conveniently said that moderate activation in Sirt1 could prevent cellular damage and lessen the possibility of all the above processes of NAD⁺ depletion followed by ATP consumption that can also consequently lead to apoptosis. Sirt1 activation can also improve glucose homeostasis and insulin sensitivity (Pfluger et al., 2008), which signifies positive impact of drug ASA and its metabolite in the said process.

A lingering question is the dose of ASA that might be involved in the observed effects. On the one hand, the clinical dose of ASA is based on its ability to control thromboxane production. The plasma half-life of ASA is less than 15 min whereas that of SA is substantially higher (Petersen et al., 1982). In addition, SA concentration in plasma varies with oral uptake and could be substantially increased (McCord and Fridovich, 1969; Williams and Hennekens, 2004; Zhang et al., 2007). The current study might shed light on ASA's unexpected beneficial actions beyond its role in the control of cyclooxygenases, including its ability to induce nitric oxide synthesis (Santanam and Parthasarathy, 2006; Nemoto et al., 2005; Alcendor et al., 2004). Our studies suggest that the effects of ASA could be mediated by MAP kinase and NF κ B signaling pathway. Thus, it can be speculated that an increase in MAP kinase signaling would basically mediate the activation of AhR, Sirt1 and redox signaling. MAP kinase is also known to regulate Nrf2 signaling pathway mainly by indirect mechanisms. Nrf2 and antioxidant response element (ARE) are responsible for both the basal and inducible expression of genes involved in antioxidant response NQO1. Sirt1 increase shows its protective role and negative effect on insulin-resistance, diabetics and vascular function. Furthermore, decrease in AKT shows increase in PGC-1 α that could regulate process of gluconeogenesis in liver (Nemoto et al., 2005). In NF κ B signaling pathway it is found that fos gene shows highest induction, which can activate tyrosine hydroxylase.

Additionally, PGC-1 α expression can also up regulate energy metabolic pathways. Literature suggests that increase in PON1 and decrease in NF κ B occurs due to Sirt1 activation (Pfluger et al., 2008; Petersen et al., 1982). This also suggests decrease in inflammatory cytokines Il6, Lrf1, Lta, Csf3, C3 and Tlr2, Tlr6 with an increase in PGC-1 α . Akt decrease indicates that it might regulate insulin levels that could inhibit apoptosis. Thus both MAP kinase and NF κ B signaling pathways might play significant role in mediating ASA effects.

5. Conclusions

Results presented in this study suggest that the benefits of aspirin might go beyond the inhibition of cyclooxygenases. The results also suggest that metabolites of aspirin/salicylate via hydroxylation reactions might be key determinants of its novel actions. Paradoxically, the generation of oxygen free radicals, generally believed to be deleterious, might be important in the actions of aspirin.

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Fig. 1.

Induction of SIRT1 & PGC-1α in HepG2 cells and mouse primary hepatocytes: HepG2 cells and mouse primary hepatocytes treated with different concentrations of ASA (0–0.25 mM) for 48 h. SIRT1 and PGC-1α were induced in HepG2 cells (A and C, respectively) and in mouse primary hepatocytes (B and D, respectively). (E) Western blot analysis of SIRT1 & PGC1α in HepG2 cells treated with ASA showed increased protein expression as compared to control. (F) Induction of SIRT1 in HEPG2 cells treated with 50 μM H₂O₂. Values are expressed as mean ± S.D. (*n*=3). Statistically significant changes between untreated and treated sets are marked as * (*P*<0.05).

Fig. 2.

Regulation of Sirt1 gene expression: (A) increase in Sirt1 gene expression in HepG2 cells treated with DHBA, (B) in HepG2 cells ASA treatment revealed increased induction in Nqo1 gene expression. SIRT1 gene expression of HepG2 cells transfected with (C) PON1 siRNA (D) AhR siRNA was analyzed. Cells treated with control siRNA were also maintained. (E) Primary hepatocytes that were treated with MAPK inhibitor SB203580 pose inhibitory effect on AhR gene expression in 50 μM and 0.1 mM ASA treated cells (F) Decrease in Sirt1 gene expression in presence of SB203580 and 50 μM ASA treated hepatocytes for 48 h compared to drug alone. All the values are expressed as mean±S.D. ($n=3$). Statistical significant differences between untreated and treated conditions are marked as * ($P<0.05$).

Fig. 3.

Confocal microscopy for detection of intracellular H_2O_2 and $O_2^{\cdot -}$: (A) HepG2 cells treated with 50 μ M, 0.1 mM ASA for 48 h showed increased number of cells stained by DCDHF-DA compared to control. HepG2 cells transfected with AhR siRNA and treated with 0.1 mM ASA did not show any stained cells as compared to HepG2 cells treated with 0.1 mM ASA. (B) HepG2 cells treated with 50 μ M, 0.1 mM ASA for 48 h showed increased number of cells stained by DHE compared to control.

Fig. 4.

Staining of mitochondria of HepG2 cells: cells were incubated with 50, 100 and 250 μM of (A) aspirin or (B) sodium salicylate followed by staining with MitoTracker Green dye as described in methods. Cells were counterstained with DAPI (blue). The cells were visualized by fluorescence microscopy (magnification, 100x).

Scheme 1.

Proposed mechanism of Aspirin action in the induction of Sirt1 gene. ASA would be hydrolyzed by PON1 to SA and the latter would be hydroxylated to DHBA by AhR mediated reactions. Redox reactions of DHBA would generate superoxide and H_2O_2 , which would induce Sirt1 among other reactions.