Sodium Antimony Gluconate Induces Generation of Reactive Oxygen Species and Nitric Oxide via Phosphoinositide 3-Kinase Activation in Leishmania donovani-Infected Macrophages

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Visceral leishmaniasis, caused by Leishmania donovani, is fatal if left untreated. The pentavalent antimony (SbV) compound urea stibamine first emerged as an effective chemotherapeutic agent against Indian kala-azar (6). Although different forms of pentavalent antimony complexes (chelates, i.e., SbV chelated to an organic backbone), namely, sodium stibogluconate (Pentostam) and meglumine antimoniate (Glucantime), are still the first choice for treatment of leishmaniasis (21, 42), their mechanism of action is still largely unknown. Previous studies indicated that sodium antimony gluconate (SAG) failed to act in immune-compromised hosts, such as patients who are suffering from AIDS or receiving immunosuppressive agents (17, 38) and immunocompromised hosts, such as patients who are suffering from AIDS or receiving immunosuppressive agents (17, 38) and severe combined immunodeficient (SCID) mice (15). Several studies have shown that endogenous interleukin-2 (IL-2) (34), IL-4 (1, 43), and IL-12 (41) influence the effectiveness of chemotherapy with pentavalent antimony. These findings are inclined to indicate the requirement of a somewhat functional T-cell compartment for SAG action. Moreover, SAG has been found to inhibit selective protein tyrosine phosphatases (Src homology 2 domain-containing tyrosine phosphatase 1 [SHP1] and SHP2) in vitro and augment cytokine signaling and responses in hematopoietic cell lines (46), suggesting the role of phosphatases and possibly other signal transduction pathways in SAG-induced control of Leishmania infection. In addition, the dose of SAG that kills the axenic amastigotes in vitro is 50 times higher than the concentration of the drug required for killing the parasite within macrophages (Mφs) (23), suggesting host cell activation as an integral component of SAG-induced antileishmanial effects. Moreover, SAG synergizes with alpha interferon (IFN-α) to activate STAT1 to kill IFN-α-resistant human cancer cell lines, like WM9 (melanoma), SW620 (colon carcinoma), U266 (multiple myeloma), MDA231 (prostate cancer), etc., in vitro (56). These studies indicate that this metalloid chelate might modulate a number of signaling events. Thus, this study has provided the first evidence that SAG treatment induces activation of some important components of the intracellular signaling pathway, which results in an early wave of ROS-dependent parasite killing and a stronger late wave of NO-dependent parasite killing. This opens up the possibility of this metalloid chelate being used in the treatment of various diseases either alone or in combination with other drugs and vaccines.

Pentavalent antimony complexes, such as sodium stibogluconate and sodium antimony gluconate (SAG), are still the first choice for chemotherapy against various forms of leishmaniasis, including visceral leishmaniasis, or kala-azar. Although the requirement of a somewhat functional immune system for the antileishmanial action of antimony was reported previously, the cellular and molecular mechanism of action of SAG was not clear. Herein, we show that SAG induces extracellular signal-regulated kinase 1 (ERK-1) and ERK-2 phosphorylation through phosphoinositide 3-kinase (PI3K), protein kinase C, and Ras activation and p38 mitogen-activated protein kinase (MAPK) phosphorylation through PI3K and Akt activation. ERK-1 and ERK-2 activation results in an increase in the production of reactive oxygen species (ROS) 3 to 6 h after SAG treatment, while p38 MAPK activation and subsequent tumor necrosis factor alpha release result in the production of nitric oxide (NO) 24 h after SAG treatment. Thus, this study has provided the first evidence that SAG treatment induces activation of some important components of the intracellular signaling pathway, which results in an early wave of ROS-dependent parasite killing and a stronger late wave of NO-dependent parasite killing. This opens up the possibility of this metalloid chelate being used in the treatment of various diseases either alone or in combination with other drugs and vaccines.

MATERIALS AND METHODS

Antibodies and other reagents. Anti-β-actin monoclonal antibody (MAb) was purchased from Sigma (St. Louis, MO). Anti-tumor necrosis factor alpha (TNF-α) MAb and an Opt enzyme immunoassay kit for assay of TNF-α were obtained from BD Biosciences (San Diego, CA). Anti-PI3K and anti-phospho-PI3K (p85) were obtained from Santa Cruz Biotech, Inc. All other antibodies were purchased from Cell Signaling Technology, Beverly, MA.
SAG INDUCES REACTIVE OXYGEN SPECIES AND NITRIC OXIDE

ABSTRACT

Parasites, animals, and cell culture. Leishmania donovani MHOM/IN/1983/AG83 (50) were used. The aim of this investigation was to study the effect of SAG on the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in M. donovani promastigotes. The method to study ROS generation was by using the cell-permeable probe DCFDA, while RNS were measured by Griess reaction. SAG induced ROS and RNS generation in promastigotes at a concentration of 10 μg/ml. The level of ROS increased with time, reached a peak at 3 h and decreased gradually thereafter. Similarly, there was a slight enhancement of NO generation with time in uninfected M. donovani. Since ROS and NO are known to play a role in the pathogenesis of certain diseases, this study provides new insights into the mechanisms of SAG action on promastigotes. The results suggest that SAG may be a potential chemotherapeutic agent for the treatment of visceral leishmaniasis.

RESULTS

SAG-induced generation of ROS and NO in macrophage and control of intracellular parasites. Since ROS and NO are the two major microbicidal molecules (28), we examined whether the antileishmanial effects of SAG were dependent on these free radicals. It was observed that the amoestigotes grew until 24 h after infection in the SAG-untreated M. donovani, whereas in SAG-treated M. donovani the amoestigote count steadily declined (Fig. 1A), suggesting an early control of the parasite load by SAG.

Since pentavalent antimonial treatment in vitro induces generation of sodium peroxymonocupric ion in human polymorphonuclear cells (48), we studied the ability of SAG to induce ROS in uninfected M. donovani and I-M. donovani. We observed that ROS generation was enhanced significantly (P < 0.001) in uninfected M. donovani upon SAG treatment. The peak was attained at 3 h, and the level decreased gradually thereafter (Fig. 1B). Similarly, there was a slight enhancement of NO generation with time in uninfected M. donovani but this was significantly (P < 0.001) enhanced upon SAG treatment (Fig. 1C). When I-M. donovani were treated with SAG, there was early ROS and late NO generation, which was essentially comparable to observations with SAG-treated uninfected M. donovani (Fig. 1B and C). However, there was a slight right shift of the peak of ROS generation, the cause of which is not known (Fig. 1B). Interestingly, as with stalk-elicted M. donovani, SAG could induce both ROS and NO generation in resident peritoneal M. donovani and could induce high levels of ROS generation in P388D1 cells (data not shown).

To ascertain that ROS is indeed involved in the early-hour killing of L. donovani, I-M. donovani were treated with NAC, a potent...
scavenger of ROS, 1 h prior to SAG treatment; this inhibited the SAG-mediated killing of intracellular \textit{L. donovani} at 6 h (i.e., when SAG-mediated ROS generation was highest and NO generation was negligible in I-M\text{\textregistered}Ms) by more than 60% (Fig. 2A). Similar data were also obtained with tocopherol at this time point (unpublished data). For our subsequent experiments concerning ROS generation, we have taken the 6-h posttreatment time point because ROS generation peaked at this point in I-M\text{\textregistered}Ms in response to SAG treatment (Fig. 1B).

Since a significant level of NO was produced at 24 h and 48 h (Fig. 1C) in SAG-treated I-M\text{\textregistered}Ms, we assessed the intracellular parasite numbers in the presence or absence of \textit{t}-NMMA at these time points. Significant (\(P < 0.001\)) inhibition of SAG-mediated parasite killing occurred in the presence of \textit{t}-NMMA at 24 h post-SAG treatment, the action being more pronounced at the 48-h time point (Fig. 2A). The role of iNOS2 was demonstrated by the lack of an antileishmanial effect of SAG in iNOS-deficient M\text{\textregistered}s at a later phase of infection in vitro (Fig. 2B). It has been shown earlier that CD40-induced expression of iNOS2 is mediated by p38 MAPK activation (31). Therefore, it is quite possible that the SAG-induced NO generation is also dependent on signaling intermediates.

Inhibitors of PI3K, PKC, Ras, and ERKs strongly inhibited SAG-induced ROS generation, while inhibitors of PI3K and p38 MAPK strongly inhibited SAG-induced NO generation. Since ROS generation is reported to involve Ras (54), PI3K (14), PKC (55), and ERKs (14, 54), we tested whether different inhibitors of these intermediates alter the SAG-induced ROS production in M\text{\textregistered}s. We observed that treatment of I-M\text{\textregistered}Ms with inhibitors of PI3K (i.e., wortmannin), PKC (i.e., calphostin C), Ras (i.e., mevastatin), or MEK (i.e., U0126) prior to SAG treatment strongly inhibited the SAG-induced ROS generation (Fig. 3, top). Taking the SAG-induced increase in ROS generation over the untreated control value as 100%, the extents of inhibition of ROS generation were \(\sim 58.5\%\) with wortmannin, \(\sim 74.16\%\) with calphostin C, \(\sim 55\%\) with mevastatin, and \(\sim 71\%\) with U0126. On the other hand, the inhibitor of p38 MAPK (i.e., SB203580) only marginally (\(\sim 16.6\%\)) inhibited the SAG-induced ROS generation. This study was carried out at 6 h because maximum ROS generation was observed for I-M\text{\textregistered}Ms at this time point.

The p38 MAPK has widely been reported to be involved in NO generation (3, 25, 31), and activation of p38 MAPK is reported to require the involvement of PI3K (25, 30, 52). Therefore, we tested with inhibitors of PI3K and p38 MAPK to determine whether SAG involves this pathway to induce NO production in M\text{\textregistered}s. It was observed that treatment of I-M\text{\textregistered}Ms with wortmannin or SB203580 prior to SAG treatment almost completely inhibited the SAG-induced NO generation at 24 h and also inhibited intracellular parasite killing by \(\sim 94\%\) or \(\sim 99\%\), respectively (Fig. 3, bottom). On the contrary, pretreatment with inhibitors of PKC, Ras, and ERKs had only minor inhibitory effects (\(\sim 5\%\), \(\sim 14.8\%\), and \(\sim 18.9\%\), respectively) on SAG-induced NO production. This study was also conducted at the 48-h time point, and the results obtained were essentially similar to those obtained at the 24-h time point. However, the result for the 24-h time point has been presented because the difference in numbers of intracellular \textit{L. donovani} parasites between untreated and SAG-treated M\text{\textregistered}Ms was maximal at this time point. Altogether, these data suggest that

**FIG. 1.** SAG-induced generation of ROS and NO in M\text{\textregistered}s and killing of intracellular parasites. Uninfected M\text{\textregistered}s and M\text{\textregistered}Ms infected with \textit{L. donovani} (I-M\text{\textregistered}Ms) were either kept untreated or treated with SAG for different durations. (A) Intracellular parasite numbers were measured for I-M\text{\textregistered}Ms in the absence or presence of SAG. (B) ROS generation was measured with DCFDA; uninfected M\text{\textregistered}s and I-M\text{\textregistered}Ms showed significant increases in ROS generation at 3 h and 6 h post-SAG treatment, respectively, compared with levels for corresponding untreated controls (\(*, P < 0.001\)). (C) Nitrite was measured using Griess reagent in cell-free culture supernatants of SAG-treated uninfected M\text{\textregistered}s; I-M\text{\textregistered}Ms showed significant increase in nitrite generation compared to untreated counterparts (\(*, P < 0.001; **, P < 0.005\)). All results are presented as means \(\pm\) SEM of five independent experiments.
SAG results in activation of PI3K, PKC, Ras, and MEK-1/MEK-2 for ROS generation and that it activates p38 MAPK for NO generation.

SAG treatment induced phosphorylation of PI3K, PDK1, Akt, PKC α/β1, Raf, ERKs, and p38 MAPK. After identifying the signaling intermediates required for SAG-induced ROS and NO generation, we examined the phosphorylation status of PI3K, PKC, Raf, ERKs, p38 MAPK, and other associated molecules, like phosphoinositide-dependent kinase 1 (PDK1) and Akt, that are intermediates in the p42/44
MAPK and p38 MAPK activation pathways (26, 30, 52). Since PKC α and β are known to be involved in the ERK activation pathway (7, 8), we tested the phosphorylation of PKC α/βII in this study.

It was observed that SAG treatment of I-Möbs for only 30 min induced the phosphorylation of PI3K, PDK1, PKC α/βII, Raf, ERK, and p38 MAPK. Phosphorylations of PDK1 and PKC α/βII were maximal at 30 min post-SAG treatment. The extents of phosphorylation of PDK1 and PKC α/βII following 0.5 h of SAG treatment were ~3-fold and ~2.5-fold, respectively, compared to that for the infected control. Thereafter, the phosphorylation level of PKC α/βII decreased gradually, but the PDK1 phosphorylation was sustained (at an ~2-fold-higher level than that for the infected control, even up to 24 h). Interestingly, Raf phosphorylation, which increased gradually from 30 min with an increase in duration of SAG treatment, remained high even up to 2 h of treatment and then decreased gradually. Strong phosphorylation of ERKs was observed up to 2 h of SAG treatment; it decreased.

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**FIG. 4.** SAG treatment induces phosphorylation of PI3K, PDK1, Akt, PKC α/βII, Raf, ERK, and p38 MAPK. I-Möbs were either kept untreated or treated with SAG for different durations; cell lysates were prepared, run in 10% polyacrylamide gels, and immunoblotted with MAb against whole and phosphorylated forms (labeled Phospho or P) of PI3K, PKC α/βII (phosphorylated form of α/βII and whole α only), PDK1, Raf, ERK-1/ERK-2, p38 MAPK, and Akt, with β-actin as an internal control. The phosphorylation status of each of the above molecules was expressed as the densitometric ratio of the phosphorylated form versus the expression control. Representative data of three similar experiments are presented. AG83, MHOM/IN/1983/AG83.
gradually thereafter. The extents of phosphorylation of ERK-1 and ERK-2 following 1 h and 2 h of SAG treatment were 1.5-fold and ~2-fold, respectively, compared to that for the infected control. Phosphorylation of Akt was very weak at 30 min following SAG treatment but increased gradually and reached the peak at about 6 h posttreatment, i.e., when the SAG-induced phosphorylation status of PKC was very weak. The extent of phosphorylation of Akt following 6 h of SAG treatment was ~2-fold compared to that for the infected control. Phosphorylation of p38 MAPK was evident only following 6 h of SAG treatment; it gradually increased up to 6 h and then decreased slightly but persisted even up to 24 h post-SAG treatment (Fig. 4). The peak of phosphorylation of p38 MAPK was observed to occur following 6 h of SAG treatment, and the extent was ~4.5-fold compared to that for the infected control.

To test the probable pathway for SAG-induced phosphorylation of ERKs and p38 MAPK in I-Møs, Western blot experiments were performed with inhibitors of PI3K, PKC, and Ras. We observed that pretreatment with wortmannin completely abrogated SAG-induced phosphorylation of PDK1, PKC, and ERKs at 1 h, and almost completely inhibited the SAG-induced phosphorylations at 30 min and almost completely inhibited the SAG-induced phosphorylations of Raf and ERKs at 1 h (Fig. 5). On the other hand, wortmannin pretreatment also completely abrogated SAG-induced phosphorylation of Akt and almost completely inhibited that of p38 MAPK at 6 h. Further, pretreatment with calphostin C or mevastatin strongly inhibited SAG-induced phosphorylations of Raf and ERKs at 1 h but not that of Akt or p38 MAPK at 6 h.

SAG treatment triggered TNF-α production through participation of p38 MAPK. Activation of p38 MAPK is well reported to trigger production of TNF-α (29), which is known to induce iNOS2 expression and NO generation (29). These studies prompted us to investigate the involvement of TNF-α in
the SAG-mediated NO generation due to activation of p38 MAPK.

SAG treatment of I-Møs induced TNF-α production (Fig. 6A), and this could be inhibited almost completely by pretreatment with wortmannin or SB203580. On the contrary, pretreatment with an inhibitor of PKC, Ras, or ERKs failed to show any significant inhibition (extent of inhibition was ~7.1%, ~14.6%, or ~12%, respectively) (Fig. 6A). We then investigated whether the SAG-induced NO production in I-Møs was mediated by TNF-α. It was observed that incubation of I-Møs with anti-TNF-α neutralizing Ab strongly suppressed SAG-induced NO production after 24 h and completely abrogated it.

FIG. 6. SAG induces TNF-α production from L. donovani-infected Møs through participation of PI3K and p38 MAPK. (A) I-Møs were either kept untreated (−) or treated (+) with wortmannin, calphostin C, mevastatin, U0126, or SB203580 prior to SAG treatment; cell-free culture supernatants were collected after 18 h and assayed for TNF-α by sandwich enzyme-linked immunosorbent assay. Pretreatment with wortmannin or SB203580 significantly inhibited SAG-mediated TNF-α generation (**, P < 0.001). (B) In some experiments, I-Møs was either kept untreated or treated with SAG in the presence or absence of neutralizing anti-TNF-α MAb, and resulting levels of nitrite accumulated in cell-free culture supernatants after 24 h of SAG treatment were measured. (C) Corresponding intracellular parasite numbers were also assessed. Neutralization of TNF-α significantly inhibited SAG-mediated NO generation (P < 0.005) as well as intracellular L. donovani killing (P < 0.005 at 24 h). Results presented are means ± SEM of four independent experiments.
after 48 h (Fig. 6B). Furthermore, incubation of I-Mdøs with anti-TNF-α neutralizing Ab also inhibited SAG-induced killing of intracellular L. donovani parasites partially (~60.8%), but significantly (P < 0.01), at 24 h and completely after 48 h (P < 0.005) (Fig. 6C).

DISCUSSION

The present study has provided the first insight into the involvement of signaling intermediates, namely, PI3K, PKC, and MAPKs, in SAG-mediated generation of the leishmanicidal molecules (ROS and NO). Our results show that SAG treatment alone induced both ROS and NO in murine Møs and induced two waves of killing of L. donovani amastigotes. The first phase of killing (i.e., at an early time point, around 6 h posttreatment) was due to induction of ROS, as evident from the sensitivity to tocopherol (data not shown) or NAC, while the second wave of killing (i.e., at later time points, 24 h and 48 h) was mediated by NO generation, as evident from the sensitivity to l-NMMA and failure of SAG to inhibit killing of intracellular L. donovani parasite replication in peritoneal Møs from iNOS−/− C57BL/6 mice at 24 h posttreatment. Both ROS and NO are known to be involved in parasite killing in the early stage of leishmanial infection in mice, whereas NO alone is involved in the late phase (39).

Since the SAG-induced phosphorylation of PKC α/βII, which occurred by 30 min posttreatment, was observed to be inhibited by wortmannin pretreatment, it is possible that SAG treatment resulted in rapid phosphorylation of PI3K, which is a well-known activator of PDK1 (9, 27) that in turn activates several PKC isotypes (9, 27). Inasmuch as wortmannin, calphostin C, and mevastatin inhibited the SAG-induced Raf phosphorylation as late as 1 h, our observations present a new mode of Raf activation which is very different from receptor activation-mediated Raf activation and recruitment to the membrane. Our results also suggest that SAG-induced activation of the PI3K-PKC-Ras-Raf pathway triggered ERK phosphorylation. Pretreatment of I-Møs with any of these inhibitors or U0126 resulted in significant inhibition (P < 0.01 for wortmannin and mevastatin and P < 0.005 for calphostin C and U0126) of SAG-mediated ROS generation, suggesting that SAG activates the PI3K-PKC-Ras-ERK pathway and thereby triggers ROS generation.

On the other hand, inhibition of PI3K or p38 MAPK strongly abrogated SAG-mediated NO production in I-Møs as well as parasite killing at ~24 h following SAG treatment. This suggested that NO production and subsequent parasite killing in response to SAG treatment involved mainly PI3K and p38 MAPK. The PI3K-Akt-p38 MAPK pathway (25) was shown to be involved in NO generation (25, 26). Activation of PKC inhibits PI3K-mediated activation of Akt (33). In our study it has also been observed that although Akt was phosphorylated 1 h following SAG treatment, the level reached a peak at 6 h posttreatment, when phosphorylation of PKC α/βII was very low. This phosphorylation could be inhibited by wortmannin pretreatment, confirming that SAG-induced prolonged activation of PI3K resulted in Akt phosphorylation. The activation of the PI3K-Akt pathway by SAG seemed to trigger phosphorylation of p38 MAPK that was almost completely abrogated by wortmannin pretreatment. The PKC-Ras pathway played a minimal role in SAG-induced p38 MAPK phosphorylation, as it was insensitive to pretreatment with calphostin C or mevastatin.

L. donovani infection has been reported to increase protein tyrosine phosphatase activity, mainly that of type SHP1 (5, 44–46), which might contribute to dysregulation of protein tyrosine kinase-dependent signaling events and Mø deactivation (44). SHP1 might directly dephosphorylate ERKs (45) and regulate activation of other important signaling molecules, like PI3K (12). Thus, inhibition of SHP1 by SAG might indirectly favor the tyrosine phosphorylation of PI3K and might thereby help in activating both the PI3K-PKC-Ras-Raf-ERK-1/ERK-2 pathway for ROS generation and the PI3K-Akt-p38 MAPK pathway leading to NO generation.

The p38 MAPK has been shown to induce TNF-α production (29), which in turn induces iNOS2 expression and subsequent NO generation (29), as suggested by the inhibition of NO generation and parasite killing by treatment with anti-TNF-α neutralizing Ab. The SAG-mediated TNF-α generation could be inhibited by wortmannin or SB203580 pretreatment, indicating that SAG-induced activation of the PI3K-p38 MAPK pathway not only activates NO-mediated parasite control but also reinforces the mechanism by triggering endogenous TNF-α that controls the parasite in an autocrine manner. Indeed, the SAG-induced antileishmanial activity is reduced in TNF-α knockout mice (35). In addition, since SAG up-regulates IFN-γ receptors in both uninfected and L. donovani-infected THP-1 cells, as well as in monocytes derived from kala-azar patients treated with SAG (13), it is quite possible that SAG influences the host’s antileishmanial defense by altering IFN-γ responsiveness. Indeed, SAG fails to act in IFN-γ knockout mice (40), and in the case of chronic, nonhealing murine cutaneous leishmaniasis, treatment with SAG plus IFN-γ induces much higher levels of iNOS2 transcripts in footpad tissues than SAG or IFN-γ alone (28). We have also observed that SAG and IFN-γ synergize to produce high levels of NO in Møs (unpublished data). A combination of SAG and IFN-γ is also known to be therapeutically much more effective than SAG alone in the treatment of visceral leishmaniasis (37). We have further observed that SAG triggers production of IL-12 in both uninfected Møs and I-Møs (unpublished data). IL-12 is known to induce Th cells to produce IFN-γ, which in turn activates Møs to produce TNF-α and, subsequently, NO. This might explain the failure of SAG to act in IL-12 knockout mice, as reported in earlier studies (41). Furthermore, an earlier study showed that SAG treatment of infected mice imparted resistance to reinfection whereas SAG treatment prior to infection imparted partial resistance to L. donovani infection (10). All of these results may explain the requirement of T cells for the activity of SAG in vivo (36). Interestingly, neither sodium gluconate (i.e., the organic backbone of SAG) nor inorganic SbV (SbCl5) could induce significant levels of ROS or NO generation and parasite killing in I-Møs, while SbCl5 (at 10 μg SbV equivalent) proved to be highly cytotoxic (unpublished data), suggesting that neither the backbone nor the metalloid alone has the immunomodulatory effect shown by SAG (an organic chelate of SbV). Therefore, the use of SAG in combination therapy (either with other drugs or possibly with vaccines) may prove beneficial. Moreover, our preliminary observations indicate that resistance to antimonials occurred mainly due to overexpression...
of MRPI on the host cell surface and consequent nonrejection of antimony by host cells harboring antimony-resistant strains of \textit{L. donovani} (unpublished observation), suggesting that the use of suitable resistance-modifying agents might sensitize Sb-resistant cases to SAG treatment. Further studies in this direction are being pursued.

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