Geldanamycin Leads to Superoxide Formation by Enzymatic and Non-enzymatic Redox Cycling

IMPLICATIONS FOR STUDIES OF Hsp90 AND ENDOTHELIAL CELL NITRIC-OXIDE SYNTHASE*

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The ansamycin antibiotic geldanamycin has frequently been used as an inhibitor of heat shock protein 90 (Hsp90), and this agent has been widely employed as a probe to examine the interactions of Hsp90 with endothelial nitric-oxide synthase. Geldanamycin contains a quinone group, which may participate in redox cycling. When geldanamycin was exposed to the flavin-containing enzyme cytochrome P-450 reductase, both semiquinone and superoxide (O2•−) radicals were detected using electron spin resonance. The treatment of endothelial cells with geldanamycin resulted in a dramatic increase in O2•− generation, which was independent of endothelial nitric-oxide synthase, because it was not inhibited by N-nitro-l-arginine methyl ester and also occurred in vascular smooth muscle cells. Diphenylene iodinium inhibited this increase in O2•− by 50%, suggesting that flavin-containing enzymes are involved in geldanamycin-induced O2•− generation. In the absence of cells, geldanamycin directly oxidized ascorbate, consumed oxygen, and produced O2•−. Geldanamycin decreased the bioavailable nitric oxide generated by 3,4-dihydrodiazetate 1,2-dioxide in smooth muscle cells by 50%, whereas pretreatment with superoxide dismutase inhibited the effect of geldanamycin. These findings demonstrate that geldanamycin generates O2•− which scavenges nitric oxide, leading to loss of its bioavailability. This effect is independent of the inhibition of Hsp90 and indicates that geldanamycin cannot be used as a specific inhibitor of Hsp90. In light of these findings, the studies using geldanamycin as an inhibitor of Hsp90 should be interpreted with caution.

The heat shock protein 90 (Hsp90) plays a major role as a chaperone protein and is thought to promote folding of a variety of enzymes to enhance their catalytic function and to protect against degradation of certain “client” proteins by the ubiquitin proteasome pathway (1). Among these proteins are the actin-binding protein calponin (2), certain steroid receptors (3), and a variety of signaling molecules (1). A particularly interesting role for Hsp90 is its ability to interact with the endothelial cell nitric-oxide synthase (eNOS). Current evidence indicates that the binding of Hsp90 to eNOS is important for the function of eNOS in response to growth factors, G-protein activation, and mechano transduction (4).

The ansamycin antibiotic geldanamycin is widely used as a specific inhibitor of the Hsp90, and it has been assigned several cellular functions based on studies using this compound. The role of Hsp90 in promoting tumor cell survival has led to both phase 1 and phase 2 trials of geldanamycin as a chemotherapeutic agent (5), and the National Cancer Institute has recently announced an initiative in developing analogs of geldanamycin as chemotherapeutic agents (6). Geldanamycin contains a quinone group (Fig. 1), and such molecules are well known to have redox-active properties (7). Quinones react with flavin-containing reductases and ascorbate to form semiquinone radicals that in turn reduce oxygen to form superoxide (O2•−) (8, 9). Of note, the cytotoxicity of the ansamycin antibiotics has been attributed to radical generation (10). Superoxide production by geldanamycin could be particularly important in the interpretation of studies in which it is used to study the function of eNOS. If geldanamycin is releasing either O2•− or promoting the release of this radical, physiological responses to endogenously produced NO may be lost because of the rapid reaction between NO and O2•− (11) rather than by specific inhibition of Hsp90. For example, it is well established that O2•− produced agents such as pyrogallol cause a dramatic impairment of NO-mediated vasodilation (12, 13).

In this study, we employed electron spin resonance (ESR) to examine the production of O2•− induced by geldanamycin and studied its effect on the bioavailability of NO. Our findings demonstrate that concentrations of this drug commonly employed in physiological studies produce large amounts of O2•− both in physiological buffers and when exposed to endothelial or smooth muscle cells. These results raise concerns regarding the use of this agent as a specific inhibitor of Hsp90 and raise questions regarding its use as a therapeutic agent.

EXPERIMENTAL PROCEDURES

Materials—Geldanamycin was obtained from Calbiochem and dissolved in Me2SO. Dihydroethidium was purchased from Molecular Probes (Eugene, Oregon). Medium 199 was obtained from Fisher. The spin-trap 5-(diethoxyphosphoryl)-5-methyl-1-pyrrole N-oxide (DEPMPO) was obtained from OXIS (Portland, OR) and stored at ~70 °C. Polyethylene glycol-conjugated superoxide dismutase (PEG-SOD) and diethylthiiramipentacetic acid (DTPA) were purchased from Sigma. Cyclic hydroxylamine 1-hydroxy-3-carboxy-2,2,5,5-tetramethyl-pyrrolidine hydrochloride (DD) was obtained from Alexis Corporation (Laufelfingen, Switzerland).
All other reagents were obtained from Sigma.

**Cell Culture**—Human umbilical vein endothelial cells (HUVECs) were cultured in medium 199 supplemented with 20% fetal bovine serum, heparin (16 units/ml), endothelial growth supplements (50 μg/ml), glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 μg/ml). Cells were used up to passage 3, and experiments were performed when cells had reached 90–100% confluency.

Smooth muscle cells (SMCs) were isolated from rat aortas by enzymatic digestion as described previously (26). Cells were grown in culture medium with 4.5 g/liter glucose supplemented with 10% calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin and passaged twice a week. Cells between passages 8 and 20 were used in experiments.

**Measurement of Cellular Superoxide Production**—To determine intracellular O$_2^\cdot$ in intact HUVECs, we used dihydroethidium, a cell-permeant dye that is oxidized by O$_2^\cdot$ to yield fluorescent ethidium bromide that intercalates with nuclear DNA (14, 15). This dye has been shown to specifically detect O$_2^\cdot$ in endothelial cells (16). Cells were rinsed with phosphate-buffered saline and incubated with 10 μM dihydroethidium in phosphate-buffered saline, pH 7.4, in a light-protected environment at 37°C. After 30 min, fluorescent confocal microscopic images were obtained using identical acquisition parameters.

As a second approach to detect O$_2^\cdot$, ESR was employed using the spin-trap DEPMPO. Cells were rinsed with ice-cold phosphate-buffered saline and scraped from the plate. After centrifugation at 1800 rpm (7 min), the cells were resuspended in 500 μl of phosphate-buffered saline buffer and kept on ice.

**Nitric Oxide Measurements**—The bioavailability of NO generated by 3,4-dihydroxalidene 1,2-dioxide in smooth muscle cells was measured by the iron-dithiocarbamate method as described previously (13). Fe(DETC)$_2$ can trap NO$_x$ producing the complex NO$\cdot$Fe(DETC)$_2$, which has a specific ESR spectrum (13). Confluent SMCs grown in serum, heparin (16 units/ml), endothelial growth supplement (50 μg/ml), and streptomycin (10 μg/ml), and NADPH (0.3 mM) were used in experiments.

**NADPH Cytochrome P-450 Reductase**—Previously, it has been shown that flavin-containing enzymes can reduce geldanamycin and produce O$_2^\cdot$-peroxynitrite radicals, which in turn may rapidly react with oxygen to generate superoxide. To determine whether flavin-containing enzymes can also reduce geldanamycin and produce O$_2^\cdot$, the NADPH cytochrome P-450 reductase was used as a model flavin-containing enzyme. The ESR spectra of DEPMPO exposed to only geldanamycin (Fig. 2A) and NADPH (Fig. 2B)
showed a small background signal of DEPMPO/OH, which reflects an impurity of the DEPMPO spin-trap. The addition of NADPH cytochrome P-450 reductase to the sample containing geldanamycin and NADPH caused the formation of strong ESR signal (Fig. 2C), which was inhibited by either superoxide dismutase (Fig. 2D) or by DPI, an inhibitor of flavin-containing enzymes (Fig. 2E). Of note, the ESR signal of the probe without geldanamycin was ~10-fold smaller (Fig. 2F) than with geldanamycin (Fig. 2C), confirming redox cycling of geldanamycin with the NADPH cytochrome P-450 reductase. The ESR spectrum of geldanamycin after exposure to the NADPH cytochrome P-450 reductase without the spin-trap revealed an ESR signal compatible with a semiquinone of geldanamycin (Fig. 2G). This result demonstrates a one-electron reduction of geldanamycin by the NADPH cytochrome P-450 reductase.

A computer analysis of geldanamycin-derived DEPMPO radical adducts revealed the presence of two radical adducts (Fig. 3). The ESR spectrum of the probe with DEPMPO, geldanamycin, NADPH, and NADPH cytochrome P-450 reductase (Fig. 3A) was simulated as a combination of the DEPMPO/OOH (Fig. 3C, 67%) and DEPMPO/OH (Fig. 3D, 24%) radical adducts (Fig. 3B). This ESR spectrum contained a hidden ESR signal of the semiquinone of geldanamycin as well (Fig. 3E, 9%). The ESR signal of semiquinone was clearly seen in the probe without geldanamycin (Fig. 2G), which consisted of the DEPMPO/OOH (42%), DEPMPO/OH (58%), and DEPMPO/Cu (18%) radical adducts (Fig. 4D). These ESR signals were inhibited by superoxide dismutase (Fig. 4E) but not affected by the NOS inhibitor L-NAME (Fig. 4F). DPI, an inhibitor of flavin enzymes, decreased this ESR signal by 60% (Fig. 4G). The presence of the DEPMPO/OH radical adduct observed in these experiments probably is because of partial decomposition of DEPMPO/OOH by peroxidases such as glutathione peroxidase as described previously (18, 19).

The effect of geldanamycin on endothelial O$_2^-$ production was also studied by using dihydroethidium staining. Untreated endothelial cells showed only minor DHE staining, and this was slightly increased by L-NAME (Fig. 5, Vehicle). The effect of L-NAME on untreated endothelial cells probably is to be associated with increased oxidation of DHE because of inhibition of NO production and an increase in ambient levels of O$_2^-$.

The treatment of endothelial cells with geldanamycin significantly increased the amount of O$_2^-$ detected by DHE, and this was not
affected by L-NAME (Fig. 5, GM).

Based on these results, we concluded that redox cycling of geldanamycin in endothelial cells causes a dramatic increase in superoxide production, which is partially inhibited by DPI but not affected by L-NAME. These data indicate that geldanamycin-stimulated superoxide production in endothelial cells is not the result of eNOS.

Stimulation of O$_2^-$ Production in Vascular Smooth Muscle Cells by Geldanamycin—The above data suggest that eNOS is not a source of O$_2^-$ production after endothelial cell exposure to geldanamycin, but that redox cycling of this compound with other flavin-containing enzymes is probably an important source. To examine this further, we also studied rat aortic vascular smooth muscle cells that do not contain NOS. Our results with these cells were very similar to that observed with endothelial cells. When vascular smooth muscle cells were exposed to DEPMPO, only a minor background DEPMPO/OH signal was observed (Fig. 6A). The addition of geldanamycin to vascular smooth muscle cells led to the formation of a strong ESR signal (Fig. 6B) that consisted of the DEPMPO/OH radical adduct (Fig. 6C). This ESR signal was inhibited by superoxide dismutase (Fig. 6D). DPI also decreased this ESR signal by 50% (Fig. 6F). These experiments in vascular smooth muscle cells prove that geldanamycin does not require eNOS to produce O$_2^-$ radicals.

Geldanamycin Oxidizes Ascorbic Acid and Cyclic Hydroxylamines—Taken together, the above studies in cultured cells indicated that geldanamycin induces a strong increase in superoxide production in both vascular smooth muscle cells and endothelial cells that is partially inhibited by DPI. The fact that geldanamycin-induced O$_2^-$ production in vascular smooth muscle and endothelial cells was only partially inhibited by DPI suggests that the presence of additional pathways for the reduction of geldanamycin is not dependent on flavin-containing enzymes. It has been shown previously that ascorbate can reduce some quinones, thereby stimulating the production of reactive oxygen species (9). Therefore, we studied the reaction between ascorbate and geldanamycin as another potential source of O$_2^-$ in intact cells.

When ascorbate was added to phosphate buffer alone, a small amount of ascorbate radical was formed, probably because of auto-oxidation of ascorbate mediated by the trace impurities of transition metals in this solution (Fig. 7, A and C). The addition of geldanamycin increased ascorbyl radical forma-

![Fig. 5. Effect of geldanamycin on endothelial O$_2^-$ production.](http://www.jbc.org/)

![Fig. 6. Spin-trapping of geldanamycin-induced formation of O$_2^-$ radicals in vascular smooth muscle cells.](http://www.jbc.org/)
Geldanamycin-mediated Superoxide Formation

Geldanamycin decreased the bioavailable NO' generated by 3,4-dihydrodiazete 1,2-dioxide in smooth muscle cells by 50% (Fig. 9, A and B). The supplementation of smooth muscle cells with PEG-SOD prevented this effect and restored the normal level of NO' (Fig. 9C).

Thus, redox cycling of geldanamycin in smooth muscle cells causes inactivation of NO'. The protective effect of PEG-SOD supports a crucial role of superoxide production in the effect of geldanamycin on vascular cells.

DISCUSSION

The present experiments demonstrate that geldanamycin in concentrations commonly employed in physiological studies and pharmacologically yields both a semiquinone radical and O2' upon exposure to a model flavin-containing enzyme. In addition, geldanamycin markedly increased both endothelial and vascular smooth muscle cell production of O2'. Our studies indicate that this effect of geldanamycin is independent of nitric-oxide synthase but probably involves the interaction of this drug with both small molecule reductants and flavin-containing enzymes. Finally, our data suggest that O2' released from geldanamycin rapidly react with NO', reducing its ability to be trapped by Fe2+(DETC)2.

In 1994, Whitesell and co-workers (31) showed that the only cellular protein that bound to geldanamycin immobilized on agarose beads was Hsp90. Based on analysis of the crystal structure of the Hsp90-geldanamycin interaction, it was subsequently shown that geldanamycin binds to the residues 9–232 of Hsp90 and that this region contains a pocket that probably is involved in ATP binding and conformational regulation of the enzyme. Based on these seminal observations, the assumption has been made that geldanamycin is a specific inhibitor of Hsp90. Our current findings suggest that although Hsp90 may be the only protein to which geldanamycin binds, there may be other effects of the compound based on its ability to generate O2'. Because O2' may serve as a precursor to many other reactive oxygen species, this property of geldanamycin may result in a myriad of cellular effects shared by other superoxide-generating molecules including the oxidation of critical thiols in proteins,
production of DNA strand breakage, and depletion of cellular antioxidant defenses. Indeed, as early as 1984, Gutteridge (21) showed that Streptorign, an anasamycin antibiotic with anaminoquinoine group similar to that of geldanamycin, caused deoxyribose degradation and that this involved the formation of a semiquinone and superoxide and subsequently a hydroxyl-like radical (21). Our current findings suggest that geldanamycin has properties similar to that of Streptorign.

Our findings also have implications for studies using geldanamycin as a probe to examine the role of Hsp90 in the modulation of eNOS function. This enzyme produces NO in response to elevated cytoplasmic calcium concentrations. During the past few years, it has become apparent that factors other than calcium-calmodulin binding are involved in the activation of eNOS. Recently, it has been reported that various endothelial cell agonists stimulate the association of Hsp90 with eNOS (22). It has been suggested that this stimulation promotes the transfer of electrons through the enzyme by folding it into a catalytically active state or by stabilizing eNOS in its dimeric form. Whereas several lines of evidence support the concept that Hsp90 associates with eNOS upon the activation of the enzyme, interpretations of the functional implications of this heterocomplex formation have largely relied on studies using the apparent effect of geldanamycin on physiological responses and the interpretation that such effects are because of inhibition of NO production (4, 22–26). For example, it has been shown that geldanamycin abolishes endothelium-dependent vasodilatation in aortas (22), mesenteric vessels (23), and cerebral vessels (24). In a similar manner, the exposure of cells to geldanamycin resulted in a dramatic decrease of detectable NO (25–28). Whereas these studies may reflect an effect of geldanamycin on Hsp90, they could as well be interpreted as being the result of enhanced production of O2 by the compound, leading to oxidative degradation of NO, and a loss of its bioactivity. Our current findings, which show that geldanamycin diminishes the detectable NO released by an exogenous NO donor, supports the notion that geldanamycin can affect NO levels independent of its production by the eNOS enzyme.

The nitric-oxide synthases can produce O2 in the absence of the critical cofactor tetrahydrobiopterin or the substrate L-arginine (29, 30). This has been shown to be attributed to a critical role of tetrahydrobiopterin electron transfer from the heme iron of NO to L-arginine. In the absence of either tetrahydrobiopterin or L-arginine, electrons are transferred to molecular oxygen resulting in the formation of O2. This phenomenon has been referred to as NO uncoupling. Recently, it has been suggested that Hsp90 plays a role in the folding of eNOS, thus directing electron flow through its oxygenase domain to L-arginine and therefore critical in preventing eNOS uncoupling (4). In this prior study, the authors showed that geldanamycin increased bovine aortic endothelial cell O2 production in response to A23187 and suggested that eNOS was the source of O2 because the NO inhibitor L-NNAME seemed to prevent this effect of geldanamycin. Our current data differ from the results of Pritchard et al. (4), because we found that endothelial cell O2 production, as detected by the spin-trap DEPMPO, was increased by geldanamycin and that this was not altered by L-NNAME. Furthermore, we found that geldanamycin increased O2 in rat aortic vascular smooth muscle cells to a similar extent as observed in endothelial cells. These latter cells do not express eNOS, indicating that the effect of geldanamycin in these cells is independent of eNOS uncoupling. The reasons for the discrepancy between our current study and the study of Pritchard et al. (4) remain unclear but may relate to the methodology of detecting O2. The electron spin resonance method we employed is probably the most accurate approach to the detection of O2 of the currently available methods. It is also possible that in this earlier study (4), the formation of geldanamycin produced a substantial amount of superoxide causing the formation of peroxynitrite. Peroxynitrite could oxidize tetrahydrobiopterin leading to uncoupling of eNOS in an Hsp90-independent fashion.

In summary, our study provides evidence that geldanamycin can participate in a redox cycling reaction that involves reaction with flavin-containing enzymes or small molecule reductants such as ascorbate or the cyclic hydroxylamine CPH. This effect of geldanamycin is independent of its ability to inhibit Hsp90 and suggests that the compound may have many other cellular effects beyond simply preventing the function of this chaperone protein. Our present data indicate that the effects of geldanamycin in studies of nitric oxide physiology be interpreted with prudence, because this molecule not only inhibits Hsp90 and any effect it may have on eNOS but also causes oxidative inactivation of nitric oxide. Finally, the treatment of humans with geldanamycin should be approached with caution given the numerous untoward cellular effects of O2 and reactive oxygen species derived from O2.
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