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Stimulus (polyphenol, IFN-γ, LPS)-dependent nitric oxide production and antileishmanial effects in RAW 264.7 macrophages

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Abstract

The effects of interferon (IFN-γ), lipopolysaccharide (LPS), and some polyphenols as individual stimuli, as well as in various combinations on NO production in non-infected and infected macrophage-like RAW 264.7 cells were investigated, with emphasis on the NO/parasite kill relationship. In non-infected and in *Leishmania* parasitized cells, gallic acid significantly inhibited the IFN-γ and LPS-induced NO detected in the supernatant. This effect was less prominent in IFN-γ- than in LPS-stimulated cells. Interestingly, and in contrast to non-infected cells, gallic acid inhibited NO production only when added within 3 h after IFN-γ + LPS. Addition of gallic acid following prolonged incubation with IFN-γ + LPS periods (24 h) no longer inhibited, sometimes even enhanced NO release. Notably, an excellent NO/parasite kill relationship was evident from all the experiments. This study was extended to a series of polyphenols (3-*O*-shikimic acid, its 3,5-digalloylated analogue, catechin, EGCG, and a procyanidin hexamer) with proven immunostimulatory activities. Although these compounds themselves were found to be weak NO-inducers, the viability of intracellular *Leishmania* parasites was considerably reduced. Furthermore, their dose-dependent effects on macrophage NO release was determined in the presence of IFN-γ and/or LPS. Again, non-infected and infected cells differed significantly in the NO response, while inhibition of IFN-γ and/or LPS-induced NO production by the tested polyphenols strongly depended on the given time of exposure and the sequence of immunological stimuli. A strong inverse correlation between NO levels and intracellular survival rates of Leishmania parasites supported the assumption that the observed inhibition of NO was not simply due to interference with the Griess assay used for detection.

Keywords: *Leishmania*; Polyphenols; Hydrolysable tannins; Proanthocyanidins; Macrophages; Antileishmanial activity; Immunomodulation; Nitric oxide

1. Introduction

Tannins are an important class of polyphenols ubiquitously distributed in higher plants. They are not only an integral part of the human diet but are also found in traditional herbal medicines. Numerous biological and pharmacological activities have been reported for this group of secondary metabolites, including antioxidant, anti-inflammatory, antitumor, antimicrobial, enzyme-inhibiting and radical scavenging properties. These and related findings explain the increasing popularity of tannin-rich preparations as health-promoting dietary supplements and as chemopreventive agents against pathophysiological conditions, e.g. cancers and cardiovascular diseases, even though knowledge on the absorption and bioavailability of ingested polyphenols is still limited.

Compared to their antioxidant and radical scavenging properties, less attention has been paid to the immunomodulatory potential of tannins. Accumulating evidence indicates tannin-induced activation of macrophages and lymphocytes, release of cytokines and effects on signaling pathways associated with antitumor and antimicrobial effector functions. The immune system is the body's

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defense against infectious agents. Activated macrophages are part of the antimicrobial immune response, which is also due to the fact that these cells are capable of producing highly toxic molecules. The production of macrophage-derived reactive oxygen and nitrogen species plays a key role in killing microbial targets. Nitric oxides (NO) are important intra- and intercellular regulatory molecules of multiple biological functions, including macrophage-mediated cytotoxicity (Moncada et al., 1991; Nathan and Hibbs, 1991; Liew et al., 1997; Bogdan et al., 2000). Endogenously derived NO is synthesized from L-arginine by nitric oxide synthases (NOS) present in different cell types and released constantly at physiological levels (Bredt, 1999). However, activation of macrophage inducible NOS (iNOS) by immunological stimuli produce large amounts of NO, much higher than the level generated by the constitutive NOS. In the presence of superoxide anion, a series of nitric oxides might prevail including the highly reactive peroxynitrite radical, a powerful prooxidant that is also involved in the host defense mechanism. Although NO functions beneficially as an antimicrobial effector molecule in the immune protection against bacteria, parasites and viruses and regulate cell survival, the relatively high and sustained level of inducible levels of NO produced during the immune response may be harmful, as dramatically shown in septic shock. Accordingly, regulation of NO production is important for human health. In the search for therapeutic agents for treatment of NO-related diseases, polyphenols have been found to inhibit NO production (Virgili et al., 1998; Kim et al., 1999; Ishii et al., 1999; Cheon et al., 2000) and secretion of cytokines such as TNF (Habtemariam, 2000; Park et al., 2000; Okabe et al., 2001; Feldman, 2005) or IL-1 (Cho et al., 2000). TNF-α release is an essential early step in a signaling cascade leading to production of antimicrobial NO (Roach et al., 1991), thus explaining the strong interest in the development of potential TNF inhibitors.

The leishmaniases comprise a group of diseases with extensive morbidity and mortality in most developing countries. Bearing in mind the urgent need for new antileishmanial drugs, due to the limitations of the current therapeutics, we have embarked on an extended study to investigate the antileishmanial potencies of polyphenols (Kolodziej and Kiderlen, 2005). In this context, we have documented the NO-inducing potential of a broad range of hydrolysable tannins, flavan-3-ols, proanthocyanidins and related polyphenols. This apparent controversy of stimulatory and inhibitory effects on the NO production by tannins is at the moment poorly understood. While much attention is paid to molecular regulatory mechanisms, little is known about the interaction between different stimuli on NO release and, hence, their effect on intracellular parasites. The studies described below will address these issues, thereby presenting a clearer picture of the immunomodulating potentials and limits of polyphenols.

2. Results and discussion

Nitric oxide and related reactive nitrogen intermediates released in activated macrophages significantly contribute to the control of microbial pathogens, but may also be involved in some pathological conditions. Differences in the cell type, the kind of stimulus and its concentration, and the stimulation conditions are significant parameters that determine NO production in experimental models. To explore possible parameters underlying positively and negatively regulated NO productions, we studied the effects of IFN-γ, LPS, and polyphenols as separate stimuli and in various combinations on the release of NO in the macrophage-like cell line, RAW 264.7, over 72 h using the Griess assay. The experiments were performed in parallel in non-infected and in *Leishmania*-infected cells, where the NO-inducing potencies were also related to the survival rates of intracellularly residing *Leishmania* parasites. The cell viability generally exceeded 85%, indicating the absence of cytotoxicity to RAW 264.7 cells at the sample test concentrations.

2.1. IFN-γ, LPS and gallic acid-induced nitric oxide release in RAW 264.7 cells

Initial studies were carried out with gallic acid (**1**), a compound found to be a relatively potent inducer of NO formation (Kolodziej and Kiderlen, 2005). Compared with the stimuli of IFN-γ (100 U/ml; 65 μM NO), LPS (10 ng/ml; 43 μM NO) and IFN-γ + LPS (84 μM NO) for positive activation controls, the NO-inducing effect of gallic acid (**1**) (7 μM NO) was found to be only modest at the sample concentration of 50 μg/ml (250 μM) when given to non-infected murine RAW 264.7 cells using the Griess assay (Ding et al., 1988; Roach et al., 1991) (Fig. 1). When gallic acid (**1**) was added simultaneously, NO levels were substantially suppressed by ca 65% (IFN-γ), 91% (LPS) and 74% (IFN-γ + LPS). Conspicuously, the inhibitory effect of gallic acid (**1**) was stronger in LPS- than in IFN-γ-stimulated macrophages.

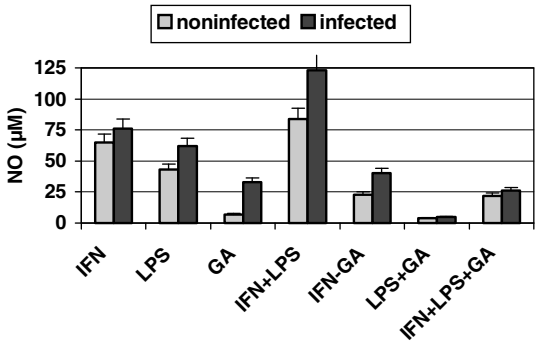


Fig. 1. NO production (μM) in non-infected and *Leishmania*-infected RAW 264.7 cells stimulated with gallic acid (**1**) (GA), positive activation controls (IFN-γ, LPS) or in combinations as indicated. NO levels in non-stimulated cells were below detection limits. The values (mean ± SEM) are derived from three independent experiments.

Using *Leishmania*-infected cells, all the stimuli (IFN-γ, LPS, IFN-γ + LPS, gallic acid (**1**)) induced a stronger NO-release when compared to that in non-infected cells. Again, gallic acid (**1**) greatly reduced the effect of the known macrophage activators when added simultaneously (Fig. 1).

Next, a set of experiments was performed in which the macrophages were pretreated with IFN-γ, LPS or gallic acid (**1**), and a second stimulus was added 30 min, 3 h or 24 h thereafter (Fig. 2). While addition of IFN-γ to LPS-primed cells or vice versa did not affect the relatively high NO levels over the time of measurements, gallic acid (**1**) again significantly reduced the induced NO levels, most notably in LPS-stimulated cells. This inhibition of NO release was the more effective, the longer gallic acid (**1**) remained in the cell culture. With gallic acid (**1**) as primary stimulus, the NO concentrations did not exceed those induced by gallic acid (**1**) alone, irrespective of the kind of standard macrophage activator that had been added as a second signal.

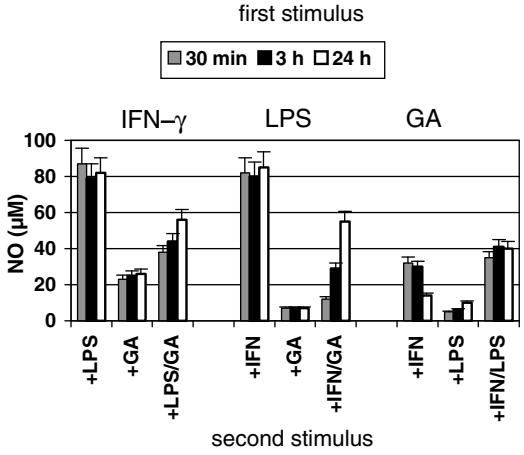


Fig. 2. Time dependent NO production (μM) in non-infected RAW 264.7 cells prestimulated with IFN-γ, LPS or gallic acid (**1**) (GA), and treated with a second stimulus added after 30 min, 3 h or 24 h. The values (mean ± SEM) are derived from three independent experiments.

A similar picture emerged when infected cells were used (Fig. 3). Conspicuously, the NO-inducing effect was clearly more pronounced than in uninfected cells. A remarkable difference was the apparent 'missing' inhibition of NO production in LPS-stimulated macrophages when gallic acid (**1**) was added 24 h after activation. Similarly, addition of IFN-γ, LPS or a combination of both to gallic acid (**1**) pre-treated macrophages after 24 h showed increased NO levels. This difference in behavior may be explicable in terms of the diseased state of the parasitized cells and an apparently prolonged antimicrobial response relative to non-infected cells, thereby overcoming any inhibitory short term effects. Support for this conjecture was provided by kinetic studies in terms of the expression of iNOS mRNA and a series of cytokines transcripts clearly demonstrating an enhanced and prolonged immune response, while relatively low and transient mRNA levels were observed in non-infected cells (Radtke et al., 2004). These findings clearly showed that the NO-inducing effects of a combination of stimuli strongly depended on the experimental conditions, especially the given time of exposure, the sequence of immunological stimulators and the physiological state of cells.

As for gallic acid (**1**), this phenol induced a relatively weak NO-stimulating activity in non-infected RAW 264.7 cells, a stronger NO release in infected cells, and an effective inhibition of LPS-induced NO production. These data do not, however, answer the question whether this was simply a matter of interference with NO-detection by the Griess-assay, or also blocking antimicrobial NO-activity, inhibiting iNOS activity or even iNOS gene expression.

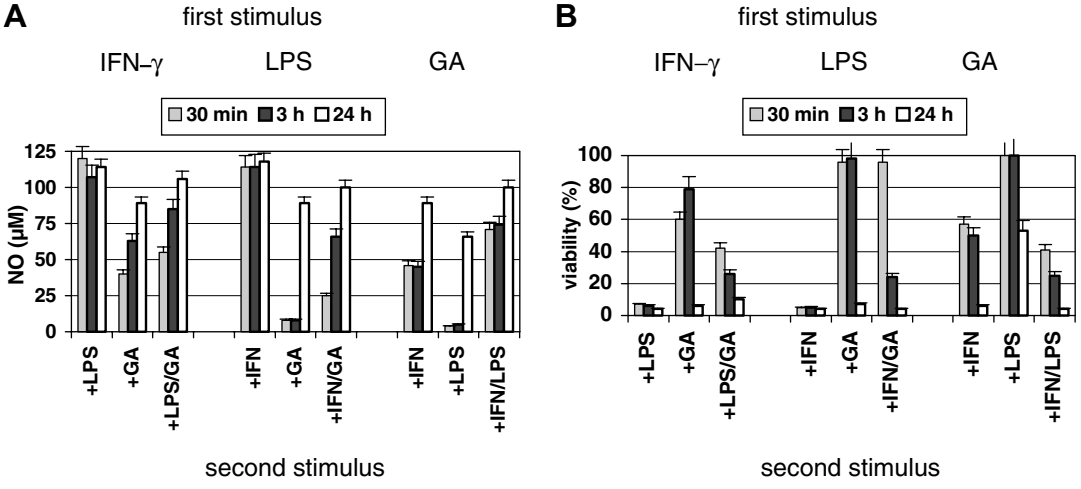


Fig. 3. (A) Time dependent NO production (μM) in *Leishmania*-infected RAW 264.7 cells prestimulated with IFN-γ, LPS or gallic acid (**1**) (GA), and treated with a second stimulus added after 30 min, 3 h or 24 h. (B) Intracellular survival of *Leishmania* parasites relative to viability in untreated cells, defined as 100%. The values (mean ± SEM) are derived from three independent experiments.

2.2. Induction of nitric oxide release in infected RAW 264.7 cells parallels parasite killing

From a medicinal point of view, the most important question was whether the reduction in measured NO concentrations effected by gallic acid (**1**) correlated with a reduction in biological activity, e.g., NO-mediated antimicrobial response. This critical issue has hitherto not been addressed in similar studies. Intracellular killing of pathogens such as *Leishmania* parasites by their macrophage hosts is thought to depend almost exclusively on NO, produced by iNOS. For the assessment of the survival rates of residing *Leishmania* amastigotes, the supernatants as a source of secreted NO were first collected measuring NO concentrations, then the host cells lysed, and the relative number of viable parasites determined using a standard MTT assay (Kiderlen and Kaye, 1990). As shown in Fig. 4, gallic acid (**1**) alone induced relatively little detectable NO (ca. 30 μM), causing only ca. 50% reduction in intracellular parasite survival. On the other hand, the combination of IFN-γ + LPS-induced ca. 125 μM NO and almost total parasite destruction. Taking into account all the given experiments, a strong inverse relationship between NO-release and intracellular survival of *Leishmania* parasites was clearly evident. Furthermore, these data indicated that the observed inhibition of NO-release by gallic acid (**1**) is not simply a matter of interference with NO-detection by the Griess-assay, but is already effective intracellularly, i.e. in the parasitophorous vacuole.

This inverse correlation was also evident, when IFN-γ, LPS and gallic acid (**1**) were given in various combinations and intervals (Fig. 4). Again, gallic acid (**1**) had the strongest inhibitory effect on NO levels induced by LPS alone, resulting in almost complete intracellular parasite survival. It was, however, effective only when given 30 min or 3 h after LPS-stimulation of cells. When given after 24 h, NO-release and intracellular parasite kill appeared enhanced relative to the effect of LPS alone over the same time of incubation (72 h). With IFN-γ, this time dependent effect of gallic acid (**1**) was not as evident.

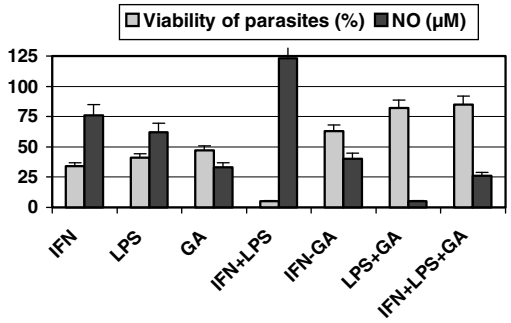


Fig. 4. Induced [IFN-γ, LPS, gallic acid (**1**) (GA)] NO production (μM) in infected RAW 264.7 cells and corresponding intracellular survival of *Leishmania* parasites relative to viability in untreated cells, defined as 100%. The values (mean ± SEM) are derived from three independent experiments.

2.3. NO-inducing potentials and antileishmanial effects of additional polyphenols

Since gallic acid (**1**) itself would not be an effective drug due to high required therapeutic levels (250 μM), these instructive results prompted further studies with a wider range of polyphenol structures, representative of simple galloylated phenols (**2** and **3**), hydrolysable tannins (**4**), flavan-3-ols (**5** and **6**) and proanthocyanidins (**7**). With NO-inducing capabilities of 4–7 μM and 5–9 μM at sample concentrations up to 100 μM in non-infected and in parasitized RAW 264.7 cells, respectively, all the tested polyphenols proved to be weak NO inducers (Fig. 5). Conspicuously, the viability of intracellular *Leishmania* organisms was considerably reduced. This deviation from a clear inverse relationship between induced NO levels and intracellular parasite survival might indicate the involvement of additional NO-independent cellular defense mechanisms or even direct antileishmanial effects of the selected compounds. An intracellular destruction of *Leishmania* parasites would imply a cellular uptake of the phenolic samples, e.g. by pinocytosis, of which no definite evidence has been reported to date. An alternative, and possibly more likely explanation is that these polyphenols have acted as scavengers for extracellular NO radicals, thus rendering this amount non-detectable for the Griess assay, while the intracellular leishmanicidal activity of NO remained unaffected. Some recent reports have demonstrated that polyphenols do have the potential to directly scavenge NO (van Acker et al., 1995; Yokozawa et al., 2000; Nakagawa and Yokozawa, 2002) and also to possess peroxynitrite scavenging activity (Haenen et al., 1997; Chung et al., 1998), thus lending support to this conjecture. Any inhibition of iNOS gene expression and enzyme activity, frequently reported for polyphenols, can not adequately explain the observed effective antimicrobial response.

In co-treatment experiments, all tested polyphenols reduced LPS-induced NO release in non-infected (data not shown) and infected RAW 264.7 cells (Fig. 6) in a dose-dependent manner, with compounds **2**, **4**, **5**, and **6** being the most effective inhibitors. It should be noted that NO production was generally higher in infected cells when compared with that in non-infected cultures. As with gallic acid (**1**), inhibition of IFN-γ-induced NO release was less evident, while the NO production induced by IFN-γ + LPS was almost unaffected by all the polyphenolic samples but compound **6**.

Regarding relationship between NO production and intracellular parasite kill, there was again a clear inverse correlation between detectable NO levels and concomitant parasite destruction. The dose-dependent reduction in NO concentrations by compounds **2–7**, particularly reflected in LPS-stimulated RAW 264.7 cells, related well with increasing intracellular survival rates of *Leishmania* parasites. On the other hand, the relatively high levels of NO released by macrophages that had been treated with IFN-γ + LPS

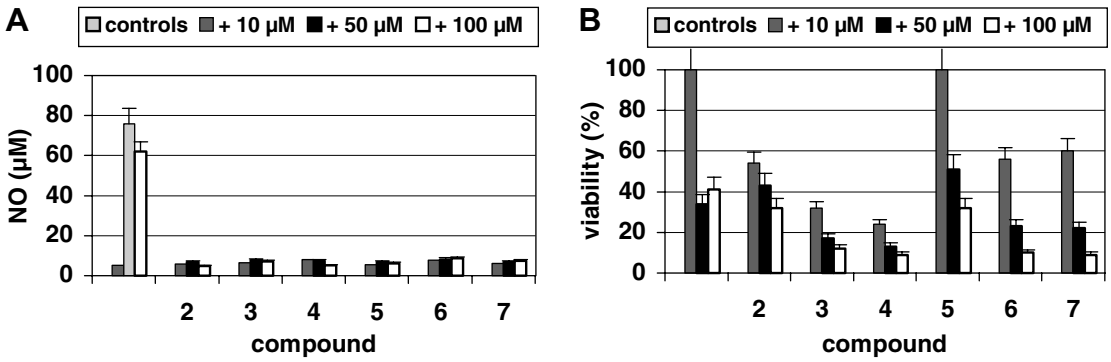


Fig. 5. (A) Dose-response data for NO production induced by compounds **2**–**7** and (B) intracellular survival of *Leishmania* parasites relative to viability in untreated cells, defined as 100%. Controls included untreated cells (negative control), IFN-γ (100 U/ml) and LPS (10 ng/ml) (positive controls), respectively. The values (mean ± SEM) are derived from three independent experiments.

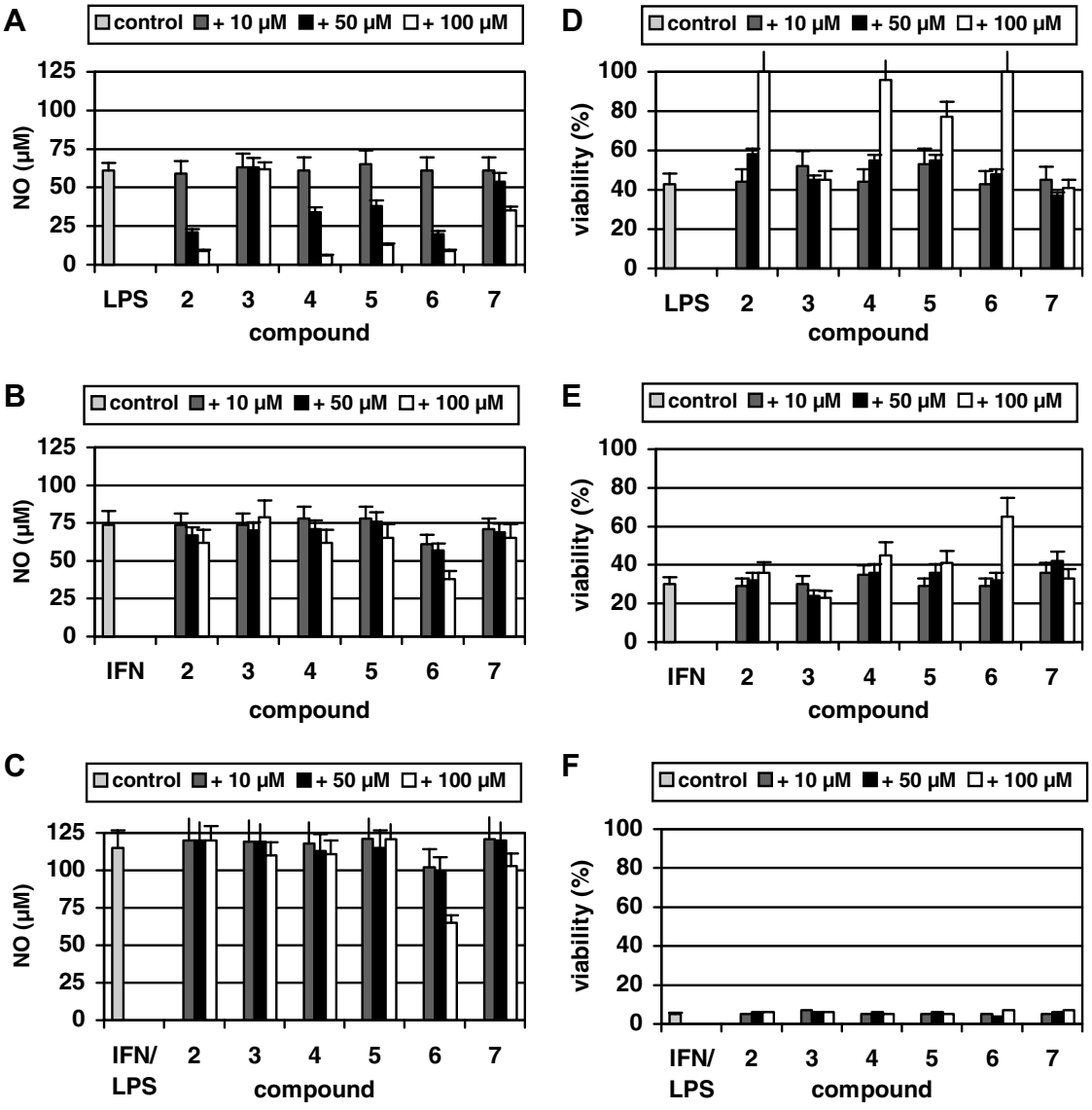
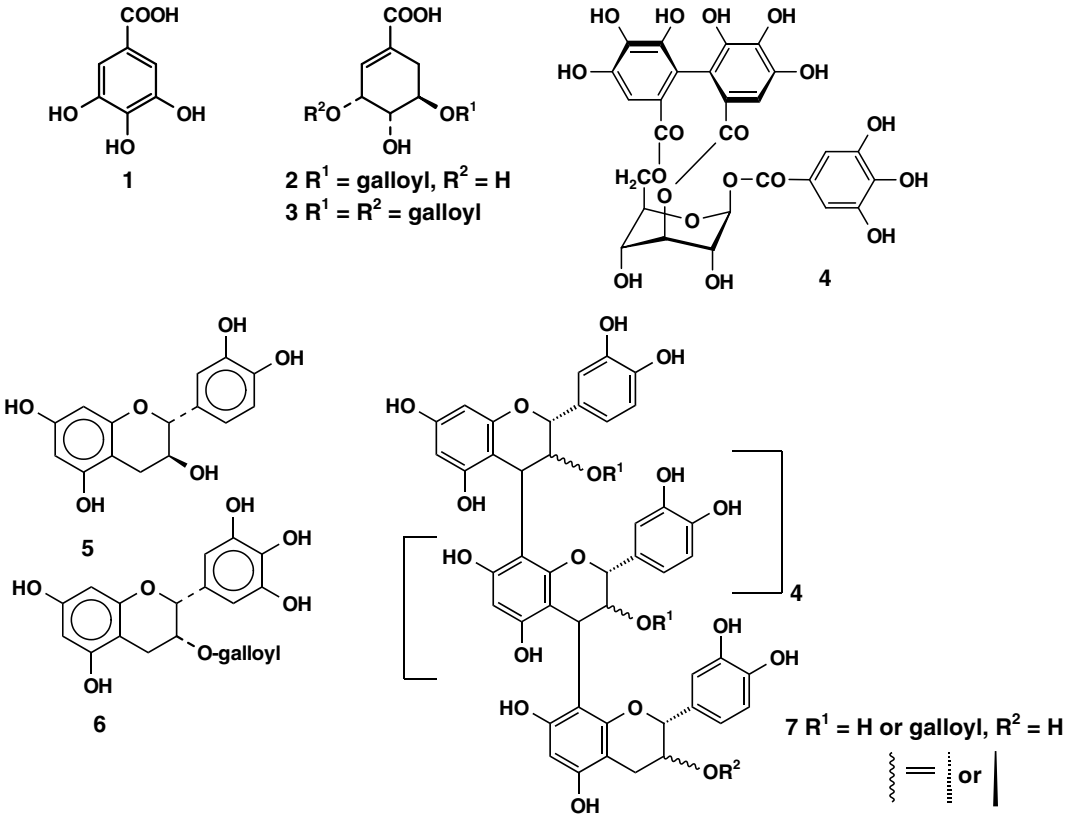


Fig. 6. Dose-response data for the induced NO production in *Leishmania*-infected RAW 264.7 cells treated simultaneously with compounds **2**–**7** and LPS, IFN-γ or IFN-γ + LPS (A–C) and reduction of viability of intracellular *Leishmania* parasites (D–F). Viability of parasites in untreated cells was defined as 100%. The values (mean ± SEM) are derived from three independent experiments.

remained largely unaffected by compounds **2–7**, as was their highly efficient killing of intracellularly residing parasites.

Conspicuously, polyphenols proved to be highly effective against *Leishmania* parasites, though only low NO levels were detectable. This may be explained by scavenging of extracellular NO under the Griess assay conditions. The NO-inducing effect was clearly more pronounced in infected than in non-infected macrophages. This apparent selectivity, in contrast to random macrophage activation, is all the more interesting in view of potentially deleterious effects of an overshooting, inadequate, NO release. Thus, a cellular regulatory principle of achieving the return to physiological NO levels from induced enhanced levels, as evident in infectious conditions, may be anticipated. Besides inhibition of signal transduction for the iNOS gene expression (Lowenstein et al., 1993; Nakayama et al., 1994; Chiu and Lin, 2005), inhibition of iNOS enzyme activity (Chan et al., 1997; Lin and Lin, 1997) may represent the underlying principal cellular and molecular mechanisms, while the NO scavenging activity of polyphenols may fortuitously contribute to the control of NO produced in excess during the immune response. It would be of great interest to learn if there is any selectivity of polyphenols



3. Concluding remarks

Although macrophages produce a variety of cytotoxic molecules for defense against microorganisms, NO is clearly considered to be the most important microbicidal effector molecules against intracellular *Leishmania* parasites and many other pathogens. The effect of polyphenols on NO release and thus on NO-mediated antimicrobial effector mechanisms seems ambiguous, as both NO-inhibitory as well as NO-inducing effects have been reported (Kolodziej and Kiderlen, 2005; Kolodziej et al., 2005). It appears as if polyphenols do have limited inducing capacities to increase NO levels, leading for instance to reduced intracellular survival of *Leishmania* parasites, though additional antileishmanial mechanisms cannot be ruled out. However, the failure of inhibition of NO production by proanthocyanidins in LPS-stimulated RAW 264.7 cells has also been reported in a single paper (Stevens et al., 2002).

in the inhibition of either pathway as well as in the scavenging of the various NO radicals.

The complexity of control mechanisms is also evident from the interference between various stimuli as shown in this study. When given simultaneously with known macrophage activators, e.g. the cytokine IFN-γ or bacterial LPS, polyphenols showed strong inhibitory effects on NO productions. Conspicuously, the inhibition concerned not only the amount of NO detectable in the Griess assay, but also its biological activity, as clearly reflected by a strong inverse NO/kill relationship. The inhibitory effect of polyphenols was stronger on LPS- than on IFN-γ-stimulated macrophage activation. Whether this involves LPS-binding to CD14 and toll-like receptor 4 or elements of signal transduction remains to be elucidated. This study provided some insight into the therapeutic significance of immunologically active polyphenols by the relationship of NO production and antileishmanial efficacy under *in vitro* conditions.

4. Experimental

4.1. Phenolic samples

All compounds tested were available in our research group and their origin, identity and purity are cited in a recent publication (Kolodziej and Kiderlen, 2005). Prior to biological activity studies, all samples were tested and found negative for endotoxin contamination (*Limulus* amoebocyte lysate method).

4.2. General procedures

General experimental procedures including cell cultures, parasites, and *in vitro* infection of macrophages with *Leishmania* parasites are fully described elsewhere (Kolodziej et al., 2001a,b; Kiderlen et al., 2001; Kiderlen and Kaye, 1990).

4.3. Assay for intracellular leishmanicidal activity

In vitro infected RAW 264.7 cells (1×10^5/100 μl/well) were mixed with 100 μl R10/well containing the indicated concentrations of either test compounds or drug standards dissolved in DMSO in a final volume of 200 μl R10 medium. Under these conditions, DMSO did not affect intracellular parasite survival. Each concentration was tested in duplicate. After incubation at 37 °C for 72 h, RAW cells were rinsed with 37 °C lysis medium and then incubated with 100 μl/well freshly prepared lysis medium for 7–20 min during which macrophage disintegration was regularly monitored with an inverted microscope. Once more than ca 95% of host cells appeared lysed, 150 μl/well post-lysis medium was added to stop SDS-lysis and to create optimal growth conditions for *Leishmania* parasites. The lysates were then incubated at 25 °C for 3–4 days to allow viable parasites to transform back to promastigotes. The relative number of viable *Leishmania*/well was determined using the MTT assay and related to that of untreated cells, defined as 100%.

4.4. Assay for cytotoxic activity against host cells

Non-infected RAW 264.7 cells were exposed to the samples test concentrations for 72 h directly in parallel to the assay for intracellular leishmanicidal activity described above. MTT was added for the final 6 h. The test compounds showed no significant cytotoxicity to RAW 264.7 cells under the experimental conditions (cell viability >85%).

4.5. Assay for NO production by activated macrophages

The murine macrophage cell line RAW 264.7 was cultured in R10 medium at 37 °C in humidified air with 5% CO_2. Cells were seeded at 1×10^5/ml in 96-well microtiter plates and were activated by incubating in R10 medium containing the test compounds in 10 μM, 50 μM or 100 μM, respectively. After 48 h, the supernatants were collected as a source of secreted NO which were quantitated by determining the nitrite concentration using the Griess assay. Aliquots of 100 μl of the supernatant were mixed with 100 μl of Griess reagents (1% sulfanilamide/0.1% naphthylethylendiamine dihydrochloride/3% H_3PO_4). After the chromophore was formed at room temperature for 5 min, absorbance was determined at 550 nm. The nitrite concentration was calculated from a standard curve generated with $NaNO_2$. The experiments were performed in duplicates.

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