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The effects of organic solvents on poly(ADP-ribose) polymerase-1 activity: implications for neurotoxicity

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Abstract. Poly(ADP-ribose) polymerase-1 (PARP-1; EC 2.4.2.30), also termed as poly(ADP-ribose) synthetase, is a key enzyme in the recognition and repair of damaged DNA. Several conditions (e.g., ischemia-reperfusion or chemical-induced injury) have been shown to overactivate PARP-1, causing neurodegeneration and necrotic or apoptotic cell death from NAD^+ and ATP depletion. In contrast, inhibitors of PARP-1 have been shown to have a neuroprotective effect by ameliorating this response. The purpose of this study was to determine the effects of three routinely used organic solvents (ethanol, methanol, and dimethyl sulfoxide (DMSO)) on the activity of purified PARP-1. A dose-response was examined with each of these solvents. A 112% and 82% increase in PARP-1 activity was observed with 15% ethanol and 20% methanol, respectively. In contrast, a near 20% decrease in the activity was observed with 4% DMSO. Kinetic analysis revealed that the maximal velocity remained unchanged with increasing concentrations of DMSO up to 20%, indicating that DMSO is a competitive inhibitor of PARP-1. Thus, PARP-1 inhibition by DMSO depends on NAD^+ concentration and in some pathological processes might be significant even at low DMSO concentrations. Our findings suggest that the interpretation of data from dose-response studies obtained when using common organic solvents may be dramatically skewed, either exaggerating the inherent toxicity of the compound or masking its potential for damage.

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INTRODUCTION

Several aspects of animal testing in toxicology may lead to erroneous conclusions when extrapolating dose-response data from animals to humans, namely differences in life expectancy, xenobiotic metabolism, and repair enzymes among species and strains of test animals (Kacew 2001, Kacew and Festing 1996, Kacew et al. 1995, 1998). In addition, the toxicity of a compound can be masked by the choice of solvent used to deliver the chemical of interest (Easterbrook et al. 2001, Kontir et al. 1986). Organic solvents inhibit several key enzymes responsible for maintaining the integrity of the genome. Of these, poly(ADP-ribose) polymerase-1 (PARP-1; EC 2.4.2.30), a zinc-finger containing enzyme involved in the base-excision repair pathway, has been reported to be particularly sensitive (Banasik and Ueda 1999).

Base-excision repair enzymes correct for most types of oxidative damage and allow for repair (Boiteux and Radicella 1999). Four major steps are involved in the base-excision repair pathway: (i) recognition and excision of the oxidized base by a DNA glycosylase (Boiteux et al. 1998); (ii) cleavage of the deoxyribose--phosphate backbone by the 5'-apurinic/apyrimidinic endonuclease (Fritz 2000); (iii) site-recognition by PARP-1 and targeting of a DNA polymerase for nucleotide insertion (Dantzer et al. 1999); and (iv) restoration of the intact DNA by a DNA ligase and PARP-1 (Creissen and Shall 1982, Oikawa et al. 1980, Tomkinson et al. 2001). Reactive oxygen species formed during normal aerobic cellular metabolism generate a variety of DNA lesions including modified bases, abasic sites, and single strand breaks with blocked 3' termini (Peskin 1997). If left unrepaired, these damages may contribute to a number of detrimental processes, including cancer, neurodegenerative diseases, and aging (Alam et al. 1997, Dreher and Junod 1996, Perez-Campo et al. 1998, Strosznajder et al. 2000, Strosznajder et al. 2003). It is estimated that as many as 10 000 oxidative "hits" occur per cell per day in the human genome. In rodents, the number of "hits" is ten-fold higher (Beckman and Ames 1997). Quantitatively, the base-excision repair enzyme system is the most important pathway for removing these kinds of lesions and maintaining the integrity of the genome (Cadet et al. 2000). In addition to its participation in base-excision repair, PARP-1 is implicated in several other biological processes including: cell differentiation (Caplan and Rosenberg 1975, Ueda et al. 1995), cell cycle control (Dantzer et al. 1998, Earle et al. 2000), transformation (Masutani et al. 2001), transcription (Simbulan--Rosenthal et al. 1996), alteration of chromatin architecture (Earle et al. 2000), and necrosis or apoptosis (Ha and Snyder 1999, Ha et al. 2002). The role of PARP-1 in determining the fate of a cell to either undergo necrosis or apoptosis is a primary interest in toxicology and risk assessment. Chemical insults can overactivate PARP-1 causing a depletion of its substrate β-nicotinamide adenine dinucleotide (NAD^+) and then ATP, leading to a major energy deficit and subsequently to cell death (Ha and Snyder 1999). PARP-1 overactivation appears to result primarily in necrosis while apoptosis is associated with caspase-dependent cleavage of PARP-1, which may conserve energy needed for the apoptotic process (Simbulan-Rosenthal et al. 1999). More recently, apoptosis-inducing factor has been shown to regulate apoptosis *via* PARP-1, independently of caspases (Cole and Perez-Polo 2002, Yu et al. 2002). Based on these findings, it was the purpose of this study to determine the effects of three commonly used solvents in toxicology, i.e., ethanol, methanol, and dimethyl sulfoxide (DMSO), on PARP-1 activity.

METHODS

Materials

PARP-1 was purified from bovine thymus as previously reported (Yoshihara et al. 1978). [*Adenosine*--U-^{14}C]NAD^+ was obtained from Amersham International plc (Buckinghamshire, UK). Calf thymus DNA (type I, highly polymerized) was from Sigma Chemical Co. (St. Louis, MO, USA), NAD^+ from Kohjin Co. Ltd. (Tokyo, Japan), and DMSO, ethanol, and methanol were from Nacalai Tesque Inc. (Kyoto, Japan). All other compounds were of the best quality commercially available (usually >98% pure, according to the manufacturers' information sheets).

PARP-1 activity assay

The activity of PARP-1 was measured as previously described (Banasik et al. 1990). Briefly, PARP-1 activity was assayed by measuring the radioactivity incorporated from [*adenosine*-U-^{14}C]NAD^+ into trichloroacetic acid (TCA)-insoluble material. The reaction mixture (200 μl) contained 100 mM Tris/HCl (pH 8.0), 10 mM

$MgCl_2$, 5 mM dithiothreitol, 33 μg/ml DNA (sheared by sonication 10 times for 10 s), 200 μM NAD^+, including [*adenosine*-U-^{14}C]NAD^+ (~107 000 cpm), and, if any, an organic solvent. The reaction was initiated by addition of PARP-1 (0.93 μg) to the reaction mixture, carried out for 10 min at 37°C, and stopped by the addition of 0.8 ml of ice-cold 20% TCA. After standing on ice for 30 min, protein-bound [^{14}C](ADP-ribose)$_n$ was collected on a nitrocellulose filter (pore size, 0.45 μm; Millipore Corp., Billerica, MA, USA) and washed 5 times with 5% TCA. After drying the filter, the acid-insoluble ^{14}C was determined by the liquid scintillation method, in the mixture of 0.5% 2,5-diphenyloxazole and 0.03% 1,4-bis(4-methyl-5-phenyloxazol-2-yl)benzene in toluene, using a LS 5000TD liquid scintillation counter (Beckman Instruments Inc., Fullerton, CA, USA). The pH of the buffer was adjusted in a 1.0 M stock solution at 20°C.

Control (usually 5 000-6 000 cpm incorporation) was the mean of duplicates with no test compound added. Effects of organic solvents were examined in, at least two independent experiments, the difference between them being <2%, and the average values were plotted. PARP-1 activity and kinetics were calculated and graphed using GraphPad Prism version 3.00 for Windows (GraphPad Software Inc., San Diego, CA, USA).

Kinetics study

Kinetics or mode of inhibition of PARP-1 by DMSO was studied at micromolar concentrations of NAD^+. The assay conditions were the same as the standard ones except for varying concentrations of [*adenosine*--U-^{14}C]NAD^+ (231 400 cpm), 1.24 μg of the enzyme, and incubation at 25°C for 30 s.

RESULTS

Dose-response of organic solvents and PARP-1 activity

Three commonly used solvents employed in toxicological studies for water-insoluble compounds are the alcohols, ethanol and methanol, and DMSO. A dose-response of PARP-1 activity for ethanol and methanol is shown in Fig. 1.

The activating effect of ethanol was maximal at 15%, resulting in a 112% increase in PARP-1 activity above control values. Methanol exhibited an activating effect on PARP-1 albeit the maximal increase above control values was 82% at the concentration of 20%. On the other hand, ethanol and methanol, at the concentration of 50%, exhibited 100% and 99% inhibition, respectively (data not shown). A dose-response with DMSO revealed an inhibitory effect on PARP-1 activity (Fig. 2). In contrast to the activating effect of alcohols at lower concentrations, a near 20% decrease in PARP-1 activity was observed with a 4% DMSO solution. At the concentration of 50%, the inhibition by DMSO was 98% (data not shown). The half-maximal inhibitory concentration

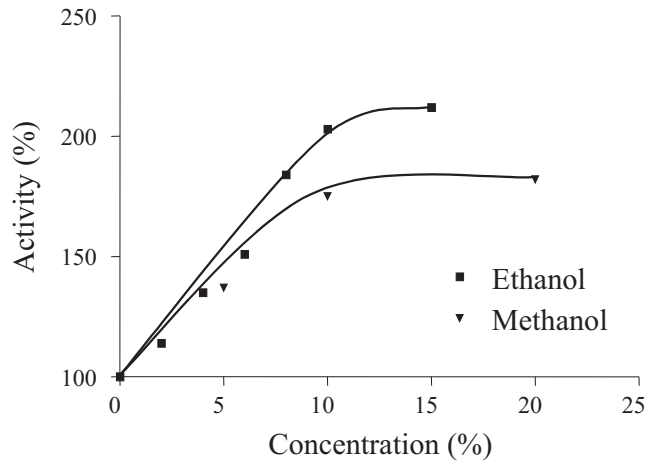

Fig. 1. The effects of ethanol and methanol on the activity of PARP-1. Values are plotted as the increase in activity above controls (100% activity). Standard reaction conditions (200 μM NAD^+, 0.93 μg of PARP-1, 37°C, 10 min).

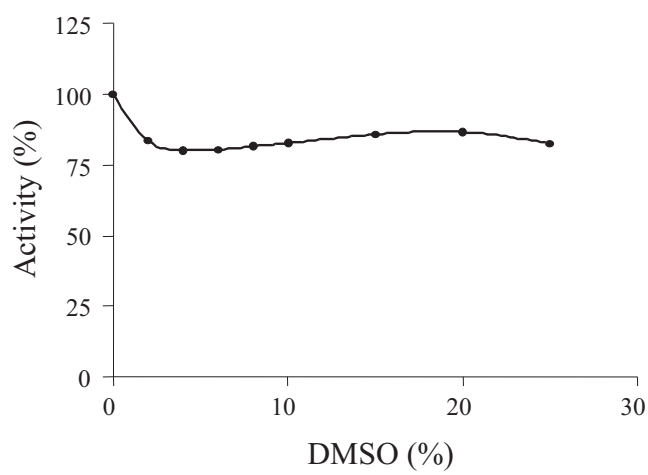

Fig. 2. The effect of DMSO on the activity of PARP-1. Values are plotted relative to the control activity. Standard reaction conditions (200 μM NAD^+, 0.93 μg of PARP-1, 37°C, 10 min).

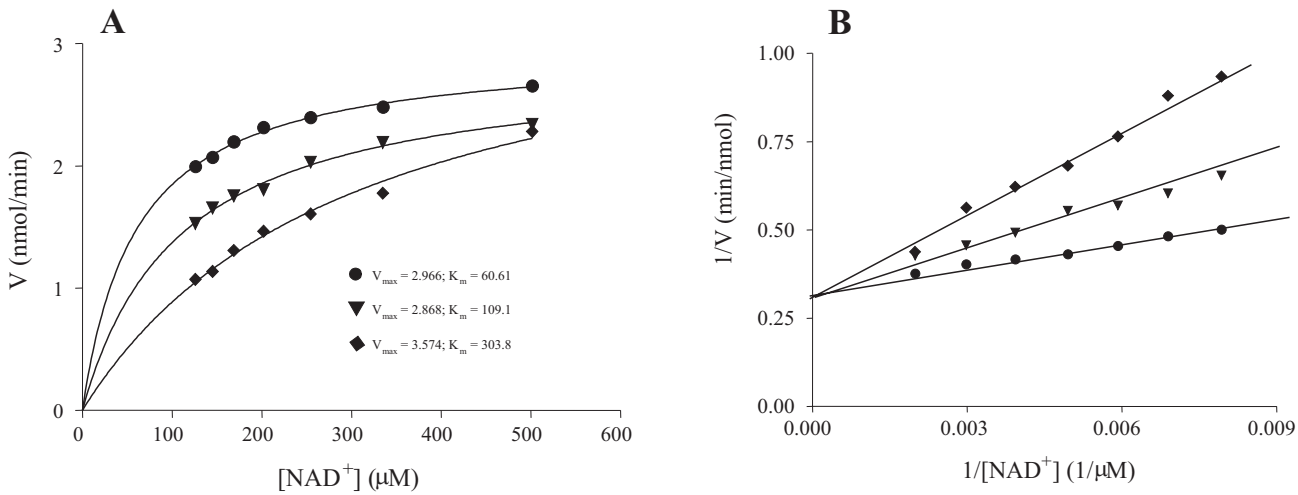

Fig. 3. Kinetics of PARP-1 activity with DMSO. (A) ● 0% DMSO, ▼ 2% DMSO, ◆ 20% DMSO; (B) Lineweaver-Burk plot. Modified reaction conditions (1.24 μg of PARP-1, 25°C, 30 s).

(IC_{50}) values of ethanol, methanol, and DMSO were graphically determined from titration curves. As shown in Table I, ethanol and methanol were strongly inhibitory at concentrations above 28% and 30%, respectively. The IC_{50} value for DMSO was 34%.

Mode of inhibition for DMSO on PARP-1 activity

Michaelis-Menten plots revealed that the maximum velocities were relatively constant with increasing concentrations of DMSO (Fig. 3A). However, the Michaelis constant (K_m) increased with increasing concentrations of DMSO, approximately 5-fold higher with 20% DMSO *versus* 0% DMSO. This pattern of inhibition is consistent with competitive inhibition and was confirmed by the Lineweaver-Burk plot (Fig. 3B). Fur-

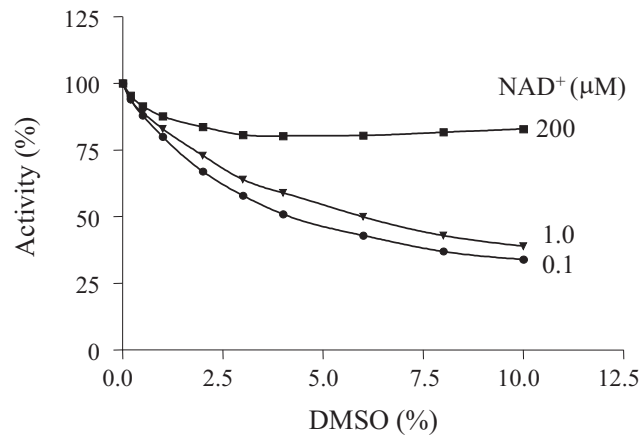

Fig. 4. The effect of DMSO on PARP-1 activity at different NAD^+ concentrations. Standard reaction conditions (■ 0.93 μg of PARP-1, 37°C, 10 min) except for NAD^+ (▼ ~120 000 cpm, ● ~12 000 cpm).

Table I

IC_{50} values for organic solvents and poly(ADP-ribose) polymerase-1 activity

	IC_{50} value*	
	%	M
Ethanol	28	4.801
Methanol	30	7.406
DMSO	34	4.791

*Standard reaction conditions (200 μM NAD^+, 0.93 μg of PARP-1, 37°C, 10 min). Controls contained no solvent.

Table II

IC_{50} values for DMSO and poly(ADP-ribose) polymerase-1 activity at different NAD^+ concentrations

NAD^+ (μM)	IC_{50} value*	
	%	M
0.1	4	0.564
1.0	6	0.846
200.0	34	4.791

*Standard reaction conditions (0.93 μg of PARP-1, 37°C, 10 min) except for NAD^+ concentrations. Controls contained no solvent.

ther analysis of the inhibitory effect of DMSO was conducted with different concentrations of NAD^+ (Fig. 4). As shown in Table II, the IC_{50} value of DMSO increased proportionately with increasing concentrations of NAD^+, supporting that DMSO is a competitive inhibitor of PARP-1.

DISCUSSION

Many genotoxic agents generate DNA strand interruptions directly or indirectly, which causes the activation of PARP-1 and the synthesis of poly(ADP-ribose) from NAD^+ in cell nuclei, thereby enhancing DNA repair (Dantzer et al. 1999, 2000). Under normal conditions, the levels of poly(ADP-ribose) in nuclei are very low; however, the levels of PARP-1 (about 2×10^5 molecules) are about 10-fold higher than enzymes such as DNA polymerase α and RNA polymerase II. Overactivation of PARP-1 has been shown to occur following exposure to carcinogens and non-carcinogens (Dantzer et al. 1998, Masutani et al. 2001, Ogata et al. 1980). The resultant decrease in NAD^+ can lead to an energy deficit in the cell, which results in a necrotic cell death. In contrast, PARP-1 inhibition can facilitate apoptosis following DNA damage. The implications of this duality of function in determining the cell fate following chemical exposures are critical in assessing data from dose-response studies.

Our results show that by increasing the concentration of ethanol (up to 15%) and methanol (up to 20%), a 112% and 82% increase occurs in PARP-1 activity, respectively, in a standard condition of 200 μM NAD^+. In contrast, a near 20% decrease in PARP-1 activity is observed with 4% DMSO at 200 μM NAD^+ (IC_{50} = 34%). However, at low concentrations, 1 and 0.1 μM of NAD^+, DMSO exhibited a much greater degree of inhibition (IC_{50} = 6% and 4%, respectively) (Table II). This finding may have important implications for target-organ toxicity for specific chemicals, since the concentration of NAD^+ varies in different tissues and cell types, as does the content of PARP-1.

Studies utilizing PARP-1 knockout animals or cells have demonstrated an increase in genomic instability, following exposure to a variety of chemical agents (Ménissier de Murcia et al. 1997, Oikawa et al. 1980, Trucco et al. 1998). These findings clearly demonstrate the importance of PARP-1 in the repair of damaged DNA following chemical insult. However, the low-level treatments used for determining a threshold in dose-response studies would seemingly be skewed by PARP-1 inhibition by the solvent DMSO because overactivation of PARP-1 would be attenuated, thus protecting the drop in NAD^+ levels and allowing for a prolonged DNA repair time. This notion is particularly important in the situation where the NAD^+ concentration is also markedly lowered by DNA damage.

The role of solvents at modulating the toxicity of chemicals *via* PARP-1 has been suggested indirectly. Studies evaluating the role of solvents in dermal carcinogenicity testing with the skin tumor promoter, phorbol 12-myristate 13-acetate (TPA), have shown that ethanol or methanol and TPA cause a prompt and robust papillomatous response; however, no significant difference in papilloma responses is observed between animals treated with TPA in DMSO and negative controls (Stoll et al. 2001). Additional studies utilizing apigenin, a PARP-1 inhibitor (Banasik and Ueda 1994), and TPA-induced ornithine decarboxylase activity have shown that concomitant treatment with apigenin and DMSO provides the greatest degree of inhibition *versus* mixtures of acetone and DMSO (9:1) or propylene glycol and DMSO (4:1) (Li et al. 1996). Finally, doxorubicin-induced palmar-plantar erythrodysesthesia syndrome has been successfully treated by topical treatment with 99% DMSO (Lopez et al. 1999).

A caveat to be noted in our study is the choice of an *in vitro* assay and the high concentration of both PARP-1 and the solvents used. Numerous inconsistencies can be observed between *in vitro* and *in vivo* models; however, the activating effect of alcohol on PARP-1 activity has been previously reported *in vivo* following chronic ethanol consumption (Nomura et al. 2001). In addition, DMSO has been shown to block the hepatotoxicity of both bromobenzene and chloroform, independently of their bioactivation (Lind and Gandolfi 1999). Taken together, these studies suggest that extrapolation of the results presented herein may be reflective of the ability of solvents to activate or inhibit PARP-1 at concentrations of solvents typically used for delivery of compounds *in vivo*.

CONCLUSION

We have identified three commonly used organic solvents that elicit at moderate concentrations different responses on the activity of PARP-1. These findings may serve as a potential source of confounding that may influence the interpretation of dose-response studies. PARP-1 overactivation has been clearly implicated in numerous chemical-induced pathologies for neurotoxi-

cants, and therefore, it should be included in the battery of tests used to determine neurotoxicity.

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