L-Selegline Potentiates the Cellular Poly(ADP-Ribosyl)ation Response to Ionizing Radiation

Christine Brabeck, Ragen Pfeiffer, Alan Leake, Sascha Beneke, Ralph Meyer and Alexander Bürkle


2Department of Gerontology, University of Newcastle, Newcastle upon Tyne, UK (R.P., A.L., A.B.)
Running title: Selegiline Potentiates Poly(ADP-Ribosyl)ation Response

Corresponding author’s present address:
Prof Alexander Bürkle
Chair of Molecular Toxicology
Dept of Biology
University of Konstanz
Box X911
D-78457 Konstanz, Germany
e-mail: Alexander.Buerkle@uni-konstanz.de
Tel: +49-7531 88 40 35
Fax: +49-7531 88 40 33

Number of text pages: 16
Number of tables: 1
Number of figures: 4
Number of references: 37
Number of words in Abstract: 227
   Introduction: 590
   Discussion: 1492

List of abbreviations: DAPI, 4’,6-diamidino-2-phenylindol; DTT, dithiothreitol; ECL, enhanced chemiluminescence; FADU, fluorescence-detected alkaline DNA unwinding; MAO-B, monoaminooxidase B; pADPr, poly(ADP-ribose); PARP, poly(ADP-ribose) polymerase; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; TRI, total radioactivity input

Section: Neuropharmacology

Footnote: This article is dedicated to Prof. Harald zur Hausen on the occasion of his retirement as Head of the German Cancer Research Center (Deutsches Krebsforschungszentrum), Heidelberg, with gratitude and appreciation for 20 years of leadership.
ABSTRACT

DNA strand breaks induced by alkylating agents, oxidants or ionizing radiation trigger the covalent modification of nuclear proteins with poly(ADP-ribose), which is catalysed for the most part by poly(ADP-ribose) polymerase-1 and plays a role in DNA base-excision repair. Poly(ADP-ribosyl)ation capacity of mononuclear blood cells correlates positively with life span of mammalian species. Here we show that L-selegiline, an anti-Parkinson drug with neuroprotective activity and life span-extending effect in laboratory animals, can potentiate gamma-radiation-induced poly(ADP-ribose) formation in intact cells. COR4 hamster cells were incubated with L-selegiline (50 nM) for various time periods, followed by gamma-irradiation (45 Gy). Quantification of cellular poly(ADP-ribose) levels at 10 minutes after starting the irradiation revealed significant increases (up to 1.8-fold) in cells pre-incubated with the drug for 8 hours to 7 days compared to drug-free irradiated controls. There was no selegiline-induced change in poly(ADP-ribose) levels of unirradiated cells nor in basal or radiation-induced DNA strand breaks, respectively. Surprisingly, poly(ADP-ribose) polymerase-1 protein was downregulated by L-selegiline treatment. Addition of L-selegiline to purified poly(ADP-ribose) polymerase-1 did not alter enzymatic activity. In conclusion, the results of the present study identify a novel intervention to potentiate the cellular poly(ADP-ribosyl)ation response. We hypothesize that the effect of L-selegiline is due to modulation of accessory proteins regulating poly(ADP-ribose) polymerase-1 activity and that increased cellular poly(ADP-ribosyl)ation capacity may contribute to the neuroprotective potential and/or life span extension afforded by L-selegiline.
Activation of the 113-kDa nuclear enzyme poly(ADP-ribose) polymerase-1 (PARP-1; EC 2.4.2.30) represents one of the fastest responses of eukaryotic cells to DNA strand breaks as induced by alkylating agents, oxidants or ionizing radiation (for recent reviews see: Jacobson and Jacobson, 1999; Davidovic et al., 2001; Ha and Snyder, 2000; Shall and de Murcia, 2000). PARP-1 catalyses the covalent post-translational modification of nuclear proteins – including PARP-1 itself – with highly complex, branched chains of poly(ADP-ribose) [pADPr], using NAD⁺ as substrate. A few years ago it was discovered that PARP-1 is apparently not the sole source of pADPr formation in living cells (Shieh et al., 1998) and several other polypeptides catalysing DNA strand break dependent or independent formation of pADPr have been described since then (Ame et al., 1999; Berghammer et al., 1999; Johansson, 1999). However, under conditions of DNA strand breakage 75-97% of cellular pADPr formation in murine fibroblasts is due to activation of PARP-1 (Shieh et al., 1998).

Numerous studies have established the importance of poly(ADP-ribosyl)ation for the recovery of proliferating cells from DNA damage and the role of PARP-1 as a “survival factor” (Shall and de Murcia, 2000). PARP-1⁻/⁻ mice are acutely sensitive to alkylating agents and gamma-irradiation, in line with a role of PARP-1 in DNA base-excision repair, established in cell culture experiments (Dantzer et al., 1999; Trucco et al., 1998). In addition, PARP-1 acts as a negative regulator of DNA damage-induced genomic instability (Bürkle, 2001c; Meyer et al., 2000). In a systematic comparison of cellular poly(ADP-ribosyl)ation capacity of various mammalian species, the longest-lived species studied (i.e., man) displayed maximal enzyme activity at a level 5-fold that of the shortest-lived (rat), despite identical PARP-1 protein levels in the two species (Grube and Bürkle, 1992). The observation of cellular poly(ADP-ribosyl)ation capacity being correlated with longevity of mammalian species is very much in
line with the widely held view that DNA damage plays a major role in the ageing process (Bürkle, 2001a).

Over the past decade a plethora of highly potent PARP inhibitors as well as a variety of molecular genetic approaches to inhibit or abrogate PARP-1 activity have been developed (Shall and de Murcia, 2000). In contrast, very little work has been invested so far to identify or develop substances that might potentiate cellular poly(ADP-ribosyl)ation. Here we report that the anti-Parkinson drug L-selegiline (L-deprenyl) can potentiate poly(ADP-ribosyl)ation capacity of intact mammalian cells challenged with gamma-irradiation. Selegiline has been used for the therapy of Parkinson’s disease on the basis of its monoamine oxidase B (MAO-B) inhibitory action (Gerlach et al., 1996). But in addition it was observed that administration of selegiline at doses below MAO-B inhibition (<1µM) can extend the life span of various animal species (Freisleben et al., 1994; Knoll et al., 1989; Ruehl et al., 1997; Stoll et al., 1997). Furthermore, neuroprotective effects have been proposed for selegiline, independently of MAO-B inhibition, both in vitro and in vivo (Ebadi et al., 2002; Kitani et al., 2001; Klegeris and McGeer, 2000; Maruyama and Naoi, 1999; Semkova et al., 1996). To date, the mechanisms underlying the life span-extending and neuroprotective properties of selegiline remain largely unclear, although an anti-apoptotic function of selegiline (Naoi et al., 2000) as well as modification of the activity of endogenous antioxidant enzymes by selegiline (Kitani et al., 1999) have been discussed.

The results of the present study (i) identify a novel intervention to potentiate the cellular poly(ADP-ribosyl)ation response and (ii) lead us to hypothesize that an increased cellular poly(ADP-ribosyl)ation capacity may contribute to the neuroprotective potential and/or life span extension afforded by selegiline.
Materials and Methods

Chemicals

L-selegiline (L-deprenyl) and 4’,6-diamidino-2-phenylindol (DAPI) were purchased from Sigma (Deisenhofen, Germany). Trichloroacetic acid (TCA) was from Roth (Karlsruhe, Germany). All other chemicals were obtained from Merck (Darmstadt, Germany).

Cell culture

The SV40-transformed embryonic hamster cell line COR4 (Meyer et al., 2000) was grown as a monolayer culture in Dulbecco’s modified essential medium (DMEM) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin (all from Sigma). Cultures were incubated at 37°C with 5% CO₂. For treatment with selegiline, the drug was dissolved in millipore water, passed through a sterile filter and added to standard medium at a final concentration of 50 nM. COR4 cells were exposed to the drug for time periods ranging from 2 hours to 7 days. Every second day, if applicable, cells were trypsinized and replated in fresh medium supplemented or not with selegiline, in order to prevent confluency.

HPLC-based quantification of cellular pADPr levels

COR4 cells were irradiated using a ¹³⁷Cs γ-ray source (Gamma Cell 1000, Atomic Energy of Canada Ltd) at a dose rate of 8 Gy/min at room temperature in phosphate-buffered saline (PBS; 137 mM sodium chloride, 2.7 mM potassium chloride, 1.5 mM potassium dihydrogenphosphate, 8.1 mM disodium hydrogenphosphate). Ten min after starting the irradiation cells were precipitated with 10% ice-cold TCA. After washing once in 20% ice-cold TCA and twice with 96% ethanol, cell pellets were dissolved in 1 M KOH and incubated at 60°C for 1 hour. Quantification of pADPr was performed as described by Jacobson et al.
(1984) with minor modifications. Briefly, ADP-ribose polymer was purified by a dihydroxyboronate chromatography step and enzymatically digested to nucleosides, followed by fluorescent derivatisation. Fluorescence-based detection and quantification of pADPr-specific nucleosides was carried out using reversed-phase HPLC. Statistical analysis of the results was performed using Student’s t-test. Results were considered significant at p<0.05 and highly significant at p<0.001.

Fluorescence-spectrophotometric assay for the determination of DNA concentrations

Fluorescence-spectrophotometric determination of the DNA content of the samples prepared for HPLC-based quantification of cellular pADPr was performed essentially as described by Brunk et al. (1979). Briefly, after dissolving ethanol-washed cell pellets (see above) in 1 M KOH, 30 µl-aliquots were taken out and mixed with 30 µl of 2 M morpholino-propanesulfonic acid (MOPS), respectively, resulting in a pH of 7.0. The basal fluorescence of the dilution buffer [10 mM Tris-HCl pH 7.0, 100 mM NaCl, 10 mM EDTA, 0.5 µl/ml DAPI] was measured using a fluorescence spectrophotometer (Hitachi M2000), with an excitation wave length of 360 nm and an emission wave length of 450 nm. Ten µl of the sample was added to the reaction buffer, followed by recording of the increased fluorescence due to the presence of DNA. This procedure was repeated five times. Then 10µl of TE buffer containing COR4 DNA at a known concentration ("DNA standard") was added, followed by measurement of the fluorescence. Again the procedure was repeated five times. Linear regression analysis was performed on the data points obtained from the two DNA solutions, respectively, yielding excellent correlation coefficients (about 0.999). The DNA concentration of the test sample was deduced from the ratio of the slopes and the known concentration of the DNA standard.
SDS-PAGE and Western blotting

This was performed essentially as described (Meyer et al., 2000). Briefly, proteins were extracted from COR4 cells by incubating whole cells in lysis buffer [62.5 mM Tris-HCl pH6.8, 6 M urea, 10% glycerol, 2% SDS, 5% β-mercaptoethanol] at 95°C. Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Amersham). The membrane was incubated overnight at 4°C with a monoclonal antibody C-II-10 directed against PARP-1 (kindly provided by G.G. Poirier), diluted 1:5 in PBS, 0.05% Tween 20, 5% dry milk, followed by incubation for 1 h at room temperature with a peroxidase-linked goat anti-mouse antibody diluted 1:2000 in PBS, 0.05% Tween 20, 5% dry milk. After ECL plus reaction (APB) was carried out, the membrane was exposed in a chemiluminescence imager (LAS 100, Fuji; Raytest, Straubenhardt, Germany). Band intensity was quantified using the Aida software, version 2 (Raytest).

Fluorescence-detected alkaline DNA unwinding

This was done using the fluorescence-detected alkaline DNA unwinding (FADU) procedure (Birnboim and Jevcak, 1981) in a recently developed automated format (Pfeiffer et al., manuscript in preparation). Briefly, 7x10⁴ cells per well of a 96-well plate were lysed in a detergent/urea buffer at 0°C, followed by partial unwinding of the DNA, starting from DNA strand interruptions, under controlled alkaline conditions for 10 min at 10°C and measurement of the fraction of DNA that had remained double stranded, using the fluorescence generated by the DNA intercalating dye ‘sybr green’ as a readout. Assays were done in 12-fold parallel determination. T-samples (high control) were not exposed to alkaline pH, i.e. no unwinding occurred at all. P₀ samples (from untreated cells) and Pₓ samples (from treated cells) were exposed to alkaline pH. Therefore, in P₀ samples unwinding did occur, starting from chromosome ends and any spontaneous internal strand breaks, and also in Pₓ samples, starting
additionally from damage-induced strand breaks. The fluorescence intensity of P_x samples is inversely related to the number of DNA strand breaks present at the time of lysis.

**Determination of PARP-1 activity in vitro**

This was done essentially as described (Beneke et al., 2000). Two µg of purified recombinant human PARP-1 (Beneke et al., 2000) were combined with reaction buffer [100 mM Tris-HCl pH 8.0, 10 mM MgCl₂, 1 mM DTT, 40 µg/ml histone type IIa (Sigma), 50 µg/ml of the ‘activator’ oligonucleotide GGAATTCC (Berger and Petzold, 1985), 0.2 mM β-NAD⁺ (grade V, Sigma), 370kBq/ml P³²-NAD⁺ (NEN)] and L-selegiline at the concentrations indicated in a final volume of 100 µl. Reactions were run for 10 min at 37°C and stopped by adding 100 µl ice-cold 20% TCA. Samples were vacuum-aspirated on GFC-Whatman filters and washed with ice-cold 20% TCA and then with 70% ethanol. PARP-1 activity was quantified by β-scintillation counting of acid-insoluble radioactivity, this value being expressed as a percentage of total radioactivity input (%TRI). Statistical analysis of the results was performed using the Mann-Whitney U-test. Results were considered significant at p<0.05, and highly significant at p<0.001.
Results

pADPr levels in selegiline-exposed cells following γ-irradiation. The experimental design for the determination of cellular pADPr levels is depicted in Fig. 1, panel A. After culturing COR4 cells in standard medium supplemented with 50nM selegiline for indicated time periods varying from 2h to 7d, cells were irradiated with 45 Gy. γ-Radiation was deliberately chosen as DNA-damaging treatment in order to prevent problems with toxicokinetics or drug-drug interactions that could emerge when using chemical compounds. In all cases, pADPr synthesis was allowed to take place for a fixed time period (10 min) at room temperature before cells were precipitated using ice-cold TCA. Subsequently, determinations of both cellular pADRr and cellular DNA content were carried out as described in Materials and Methods. Following irradiation of non-pretreated (i.e. control) COR4 cells with 45 Gy, pADPr levels showed a dramatic, typically around 40-fold, increase compared to unirradiated control cells, as expected. Within each set of experiments this level was defined as “1.0” (Fig. 1B and C, ‘control’). This radiation-induced increase in pADPr levels proved significantly higher (up to about 1.8-fold) in cells pretreated with 50nM selegiline for time periods ranging from 8 hours to 7 days (Fig. 1B, C), whereas shorter duration of pretreatment did not lead to statistically significant effects (Fig. 1B). A highly significant enhancement of pADPr accumulation by 1.47-fold was detected in cells exposed to selegiline for 3 days (Fig. 1C, \(p=0.0001\)). Interestingly, radiation-induced pADPr levels tended to be significantly lower after 7 days of pre-incubation with selegiline compared to 3 days (Fig. 1C, \(p[3d\text{-}vs\text{-}7d]=0.0068\)), while still remaining significantly higher than in irradiated controls (Fig. 1C, \(p[7d\text{-}vs\text{-}control]=0.014\)).
pADPr levels in unirradiated selegiline-exposed cells. To see if drug treatment *per se* could lead to increased pADPr accumulation under the chosen conditions, pADRr levels were determined in unirradiated COR4 cells following incubation with 50nM L-selegiline for three days. As expected, the levels in unirradiated cells not exposed to selegiline were very low (<1 pmole / 100 µg DNA; Fig. 2B). No significant difference between selegiline-treated cells and control cells was observed (p=0.54), thus ruling out that the results depicted in Fig. 1 might be due to an additive effect of selegiline and gamma-irradiation.

Spontaneous and gamma-radiation-induced DNA strand breakage in selegiline-exposed COR4 cells. As PARP-1 is directly activated by DNA strand breaks, the above effect of selegiline (Fig. 1) might hypothetically be due to increased numbers of breaks introduced by the standard dose of γ-radiation applied. We therefore determined the number of DNA strand breaks in selegiline-exposed COR4 cells following γ-irradiation at various doses, using the fluorescence-detected alkaline DNA unwinding (FADU) procedure (Birnboim and Jevcak, 1981) in a recently developed automated format (Pfeiffer et al., manuscript in preparation). The $P_0 / T$ ratios, reflecting the level of spontaneous DNA strand breaks (see Materials and Methods), were 88.7% for unirradiated controls and 89.4% for unirradiated selegiline-treated cultures (not significant). Upon γ-irradiation, there was a dose-dependent reduction of $P_x / P_0$ ratios (Fig. 3A, B) as expected, but again the level of DNA strand breakage in selegiline-treated cells was very similar to that of control cells at any irradiation dose tested (Fig. 3A, B). Therefore, a putative modulation of the DNA strand breakage by selegiline preincubation can be dismissed as an explanation for the drug’s effect on radiation-induced pADPr levels (Fig. 1).
PARP-1 protein levels in the presence of selegiline. Western-blotting of whole-cell extracts was performed in order to investigate the pattern of PARP-1 expression in COR4 cells exposed to 50nM selegiline for three days (Fig. 4A). Note that cells were not irradiated prior to Western blot analysis. Blots showed a single band migrating at 113 kDa specific for PARP-1 (Fig. 4A). As expected from the absence of any toxic effect of selegiline treatment, no apoptosis-related proteolytic cleavage product was observed. Quantification of the intensity of the bands at 113 kDa surprisingly revealed a 40% reduction in extracts of selegiline-incubated cells compared with controls (Fig. 4B). A parallel gel stained with Coomassie Blue demonstrated equal loading of extracts from selegiline-exposed cells and controls (Fig. 4C). The possibility that PARP-1 might be extensively automodified following selegiline treatment and therefore might not migrate into the gel is ruled out by the data shown in Fig. 2, where no increase in cellular poly(ADP-ribose) levels was detected in unirradiated cells. Thus, under the given experimental conditions, incubation of COR4 cells with 50nM selegiline downregulated cellular PARP-1 protein levels.

Activity of PARP-1 in vitro in the presence of selegiline. To investigate if selegiline can influence PARP-1 activity in vitro, purified recombinant human PARP-1 (Beneke et al., 2000) was incubated with selegiline at various concentrations (50nM, 500nM, 5µM) in a reaction buffer comprising ³²P-labelled β-NAD⁺ as a substrate and histones serving as “acceptor” proteins. PARP-1 activity was stimulated by saturating concentrations of a double stranded oligonucleotide, which is recognised by the enzyme as double strand breaks (Berger and Petzold, 1985). Enzyme activity was expressed as the percentage of total radioactive input (% TRI) converted into acid-insoluble material (Table 1, upper row). The results did not reveal any significant change in PARP-1 activity at any concentration of selegiline tested (Table 1, lower row).
Discussion

The process of poly(ADP-ribosyl)ation has been shown to be important for the repair of DNA damage in proliferating cells and for the maintenance of genomic stability, which is the basis for the role of PARP-1 as a cytoprotective “survival factor” (Bürkle, 2001b; Bürkle, 2001c; Jacobson and Jacobson, 1999; Shall and de Murcia, 2000). The previously observed positive correlation between cellular poly(ADP-ribosyl)ation capacity and species-specific life span (Grube and Bürkle, 1992) is in perfect agreement with such cellular function of poly(ADP-ribosyl)ation.

Several laboratories have observed life span-extending and neuroprotective properties of the anti-Parkinson drug L-selegiline (Ebadi et al., 2002; Freisleben et al., 1994; Kitani et al., 1999; Kitani et al., 2001; Klegeris and McGeer, 2000; Knoll et al., 1989; Maruyama and Naoi, 1999; Naoi et al., 2000; Ruehl et al., 1997; Semkova et al., 1996; Stoll et al., 1997). These effects were recorded at concentrations below MAO-B inhibition (<1 µM), implying they were mediated by alternative mechanisms. We hypothesized that upregulation of cellular poly(ADP-ribosyl)ation capacity might be a candidate mechanism.

The results of the present study provide clear evidence in favour of this assumption. The γ-irradiation-induced formation of pADPr in living hamster cells in culture was significantly potentiated when cells were pre-incubated with 50nM selegiline for a minimum of 8 hours. The effect of selegiline on cellular poly(ADP-ribosyl)ation capacity was still present after 3, 5 or 7 days of pre-incubation with selegiline. However, by 7 days some decline in the potentiation effect was observed, with radiation-induced polymer levels being significantly lower compared to 3 days of pre-incubation, while still significantly higher than irradiated controls. This indicates that the potentiation of cellular poly(ADP-ribosyl)ation capacity induced by selegiline may be a transient phenomenon.
In principle, a variety of enzymes involved in pADPr metabolism might mediate the above effect, *i.e.* the various pADPr polymerases and also pADPr glycohydrolase, the main enzyme catalysing pADPr catabolism. However, in view of the fact that PARP-1 is carrying out the bulk of pADPr formation under conditions of DNA breakage (Shieh et al., 1998) we focussed our attention on this enzyme. As PARP-1 is activated by DNA strand breaks, the question arose whether the mere incubation of the cells with selegiline will lead to DNA damage or potentiate the damaging effects of γ-radiation, which would provide a trivial explanation for the observed effect on pADPr levels. However, cellular pADPr levels in unirradiated COR4 cells were unaffected by the presence of selegiline (Fig. 2), thus making it very unlikely that selegiline itself causes DNA damage. This observation is in keeping with the absence of DNA strand break formation by the mere incubation of cells with selegiline (Fig. 3) and also with the absence of any cytotoxic effect on COR4 cells during incubation with 50nM selegiline (data not shown), since DNA damage typically leads to cell growth arrest or cell death. To definitely exclude the rather remote possibility that selegiline might act as a radiation sensitizer and potentiate the number of DNA strand breaks forming after γ-radiation, the level of DNA strand breakage was directly determined using the FADU technique. This assay yielded very similar results in selegiline-free and pretreated cells at any radiation dose tested (Fig. 3).

Very recently, it was shown that not only nuclear but also mitochondrial poly(ADP-ribosyl)ation can occur upon DNA damaging treatment of cells (Du et al, 2003), raising the possibility that selegiline might upregulate DNA strand breakage specifically in mitochondria, thereby activating mitochondrial forms of PARP. However, immunofluorescence analyses of gamma-radiation-induced poly(ADP-ribose) formation in COR4 cells (or parental CO60 cells) we have performed in the context of other projects (unpublished) or in HeLa cells (Alvarez-Gonzalez et al, 1999) consistently revealed dose-dependent formation of poly(ADP-ribose) exclusively in the nuclei, whereas we have never observed any polymer-specific
signals in the extranuclear compartment. Thus, we believe that in the COR4 cell system any putative selective action of selegiline on mitochondrial polymer formation is very unlikely to account for the effect we describe here.

In order to address the mechanisms underlying the observed effect of selegiline on cellular pADPr levels, PARP-1 expression was studied following a 3-day incubation of the cells with 50nM selegiline. Our previous experiments with transfected hamster cell cultures had shown that overexpressing PARP-1 can lead to a large increase in cellular poly(ADP-ribosyl)ation capacity (Meyer et al., 2000). On the other hand, it should be noted that naturally occurring differences in maximal PARP-1 activity of mononuclear blood cells from different mammalian species (Grube and Bürkle, 1992) or in lymphoblastoid cell lines from centenarians and controls (Muiras et al., 1998) could not be explained by differences in PARP-1 protein levels, indicating that the regulation of poly(ADP-ribosyl)ation capacity does not exclusively depend on the regulation of PARP-1 protein expression. Along these lines, western blot analysis of selegiline-exposed cells (Fig. 4) revealed downregulation of PARP-1 protein levels by about 40%, rather than upregulation. Therefore, the observed increase in cellular poly(ADP-ribosyl)ation capacity induced by selegiline cannot be explained by any putative increase in PARP-1 protein levels. On the contrary, one might even speculate that the increased cellular poly(ADP-ribosyl)ation capacity may activate some negative feedback mechanism leading to a downregulation of cellular PARP-1 protein levels. Such a scenario might also underlie the observed waning of the selegiline effect after 7 days of pre-incubation.

In the present work, the mechanism of selegiline-induced potentiation of radiation-induced cellular pADPr levels could not yet be established. We currently speculate that cofactors (e.g. accessory proteins) regulating PARP-1 activity might be involved. Downregulation of poly(ADP-ribose) glycohydrolase activity seems to be a less likely explanation, since basal pADPr levels were not increased upon 3-day exposure of cells to the drug.
This is the first report linking L-selegiline, i.e. a clinically used anti-Parkinson drug with neuroprotective and life span-extending activity, with poly(ADP-ribosyl)ation, i.e. a highly conserved cellular reaction known to be involved in cytoprotection and to be correlated with mammalian life span. While the underlying mechanism still remains to be elucidated, our present findings may have a number of implications:

Firstly, it will be important to directly address whether or not the neuroprotective effects of selegiline are mediated by upregulation of cellular pADPr levels in neuronal cells under (sublethal) stress. If so, this might define a novel therapeutic strategy, which may seem surprising and paradoxical at first glance, in view of the present mainstream tendency to concentrate on the benefits of PARP-1 inhibition under various pathophysiological conditions in an attempt to rescue (potentially lethally) damaged neurons, muscle fibres or pancreatic islet cells. The latter paradigm is based on the fact that the poly(ADP-ribosyl)ation system plays an important pathogenetic role in a number of diseases such as diabetes mellitus type 1, ischemia-reperfusion damage in brain, heart, kidney and bowel, MPTP-induced acute Parkinsonism, hemorrhagic and septic shock and chronic inflammation of the bowel (Bürkle 2001b). In all of these disease states, accumulation of DNA damage, as induced by massive release of reactive oxygen species, leads to overactivation of PARP which subsequently results in depletion of NAD⁺ and, as a consequence, of ATP pools and failure of energy metabolism. In contrast, moderate activation of PARP-1 without alteration of cellular NAD⁺ levels can have beneficial effects under conditions of stress. For example, it has been reported in a rat model of mild and transient global cerebral ischemia that PARP activation significantly contributed to survival of hippocampal neurons after reperfusion (Nagayama et al., 2000). A similar conclusion was reached in another study showing that PARP-1 mRNA transiently increased in the dentate gyrus after a brief period of global ischemia in gerbil brain or after injection of the glutamate agonist kainic acid into rat brain, suggesting a role for PARP-1 in DNA repair following mild brain injury (Liu et al., 2000). Viewed together, there
seems to exist a spectrum of PARP-mediated biological responses, ranging from protection under conditions of mild damage to enhanced cytotoxicity under conditions of severe damage (e.g. prolonged ischemia). Therefore, our present findings fit well into the global picture of the role of poly(ADP-ribosyl)ation as it has emerged to date. We speculate that pharmacological enhancement of the poly(ADP-ribosyl)ation system using selegiline - in the absence of overt pathophysiological conditions associated with cytotoxic PARP overactivation - may prove an interesting new therapeutic option that may produce beneficial effects at the level of preventive medicine.

In addition, it will be equally interesting to investigate if the life span-extending effect of selegiline depends on upregulation of cellular pADPr levels. Last not least, our previous studies on transfected cells with increased cellular pADPr levels have revealed improved maintenance of genomic stability in proliferating cells under genotoxic stress (Bürkle, 2001c; Meyer et al., 2000). If the same could be achieved by selegiline treatment, then this drug might become an interesting candidate as an adjunct to cytotoxic chemotherapy or radiotherapy, as it would “freeze” the potentially dangerous process of genomic instability, which can be induced in malignant and/or normal cells by cytotoxic chemo-/radiotherapy and is a driving force of tumour cell progression towards ever higher levels of malignancy (Bürkle, 2001c).
Acknowledgements

We thank Prof. G.G. Poirier for the kind gift of monoclonal antibody C-II-10 and we thank Drs. S. Stoll and U. Hafner and Prof. W.E. Müller for helpful discussions and collaboration on some preliminary experiments.
References


Figure legends

**Fig. 1.** pADPr levels in γ-irradiated COR4 cells pretreated or not with L-selegiline. (A) Schematic of the experimental protocol. COR4 cells were incubated in standard growth medium containing or not 50 nM L-selegiline for varying time periods from 2 hours up to 7 days as indicated. Cells were subsequently irradiated with 45 Gy and cellular pADPr levels were determined 10 min after starting the irradiation as described in Materials and Methods. (B) Time course of 2 – 24h preincubation with L-selegiline. Given are mean values (± SEM) of the fold-increase in cellular pADPr levels relative to selegiline-free irradiated controls (n= number of samples, p = significance vs control). (C) Time course of 3 – 7d preincubation.

**Fig. 2.** Basal pADPr levels in L-selegiline-exposed and control COR4 cells. (A) Schematic of the experimental protocol. COR4 cells were incubated in standard growth medium containing (+Sel) or not (-Sel) 50 nM selegiline for 72h. Cellular pADPr levels were determined as described in Materials and Methods. (B) pADPr levels are given as mean values ± SEM of triplicates. No significant differences were observed (p=0.54).

**Fig. 3.** DNA strand breakage in γ-irradiated COR4 cells pretreated or not with L-selegiline. Cells were incubated in standard growth medium supplemented or not with 50 nM L-selegiline for 3 days, followed by γ-irradiation on ice at various doses as indicated. DNA strand breaks were determined by fluorescence-detected alkaline DNA unwinding in 12-fold parallel determination as described in Materials and Methods. (A, B) Given are the mean $P_x/P_0$ values ± SEM, representing the fraction of DNA remaining double-stranded during the alkaline DNA unwinding procedure in irradiated samples (dose x) relative to unirradiated controls, as a measure that is inversely related to the number of radiation-induced DNA strand
breaks. (B) Statistical evaluation by Student’s T-test of differences between higher vs lower irradiation dose and of selegiline-incubated cells vs controls, respectively. Note that with every doubling of irradiation dose, the numbers of DNA strand breaks increase significantly, whereas at any irradiation dose used the values from selegiline-incubated cells are very similar to those of selegiline-free cells.

**Fig. 4.** Western blot analysis of PARP-1 in COR4 cells exposed or not to L-selegiline. (A) Western blot of whole-cell extracts from COR4 cells that had been incubated or not with 50nM selegiline for 72 hours. The blot was developed with monoclonal antibody C-II-10 specific for PARP-1. Extracts were loaded at three different dilutions, respectively. (B) Quantification of intensities of the PARP-1 specific bands at 113 kDa. Note that there was about 40% reduction of band intensity as a result of selegiline treatment compared with controls. (C) Coomassie blue staining of a parallel gel, confirming equal loading of lanes.
TABLE 1. Effect of L-selegiline on the activity of purified PARP-1

<table>
<thead>
<tr>
<th>Selegiline concentration</th>
<th>control</th>
<th>50nM</th>
<th>500nM</th>
<th>5µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>% TRI</td>
<td>6.48</td>
<td>5.82</td>
<td>5.93</td>
<td>4.34</td>
</tr>
<tr>
<td>p (versus control)</td>
<td>p = 0.84</td>
<td>p = 0.65</td>
<td>p = 0.37</td>
<td></td>
</tr>
</tbody>
</table>

Purified recombinant human PARP-1 (Beneke et al., 2000) was incubated with L-selegiline at various concentrations as indicated in a reaction buffer containing radioactive labelled β-NAD⁺, histones and saturating concentrations of a double stranded “activator” oligonucleotide (Berger and Petzold, 1985). Reactions were carried out for 10 min at 37°C and stopped by precipitation with ice-cold TCA. Enzyme activity was determined in 5-fold parallel assays as the percentage of total radioactive input converted into acid-insoluble radioactivity (median of %TRI, upper row). Statistical evaluation by Mann Whitney U-test revealed no significant differences of %TRI values at different concentrations of selegiline versus controls (lower row).
Brabeck et al., Fig 1

A

50 nM Selegiline (control: no selegiline)

Exponentially growing cells in culture

Varying incubation times:
2h, 4h, 8h, 24h
3d, 5d, 7d

Irradiation with 45 Gy

10 min, RT

TCA precipitation

HPLC-based quantification of pADPr

Fluorometric assay of DNA content

Cellular pADPr levels

B

Fold increase in 45 Gy-induced cellular pADPr levels

<table>
<thead>
<tr>
<th>Hours</th>
<th>control</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>p=</td>
<td>0.14</td>
<td>0.59</td>
<td>0.04</td>
<td>0.02</td>
<td></td>
</tr>
</tbody>
</table>

C

Fold increase in 45 Gy-induced cellular pADPr levels

<table>
<thead>
<tr>
<th>Days</th>
<th>control</th>
<th>3</th>
<th>5</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=</td>
<td>9</td>
<td>9</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>p=</td>
<td>0.0001</td>
<td>0.031</td>
<td>0.014</td>
<td></td>
</tr>
</tbody>
</table>
Brabeck et al., Fig 2

A

50 nM Selegiline (control: no selegiline)

Exponentially growing cells in culture

3d

TCA-precipitation

HPLC-based quantification of pADPr

Fluorometric assay of DNA content

Cellular pADPr levels

B

<table>
<thead>
<tr>
<th></th>
<th>cellular pADPr levels [pmol/100µg DNA]</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Sel</td>
<td>0.5</td>
</tr>
<tr>
<td>+ Sel</td>
<td>0.4</td>
</tr>
</tbody>
</table>
Brabeck et al., Fig 3

A

\[
\begin{align*}
\text{\textit{control}} & \quad \text{\textit{selegilene}} \\
\hline
0 & 100 \pm 2.2 & 100 \pm 1.6 & <<0.001 & <<0.001 \\
21.5 & 45.9 \pm 1.4 & 43.0 \pm 1.3 & <<0.001 & <<0.001 \\
43 & 22.7 \pm 1.1 & 25.9 \pm 1.1 & <<0.001 & <<0.001 \\
86 & 18.7 \pm 0.4 & 18.3 \pm 0.7 & 0.636 & <<0.001 \\
\end{align*}
\]

B

This article has not been copyedited and formatted. The final version may differ from this version.
Brabeck et al., Fig 4

<table>
<thead>
<tr>
<th>Lanes</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selegiline treatment</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Cell equivalents loaded</td>
<td>$2.5 \times 10^4$</td>
<td>$2.5 \times 10^4$</td>
<td>$5 \times 10^4$</td>
<td>$5 \times 10^4$</td>
<td>$10^5$</td>
<td>$10^5$</td>
</tr>
<tr>
<td>Intensity of the band at 113 kDa [arbitrary units]</td>
<td>1.4</td>
<td>2.4</td>
<td>3.5</td>
<td>5.1</td>
<td>7.2</td>
<td>12.1</td>
</tr>
</tbody>
</table>