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Expression of Antioxidant Molecules After Peripheral Nerve Injury and Regeneration

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Oxidative stress is considered to be one of the main causes of neural damage after injury. However, little is known about the changes in mRNA expression of anti-oxidant molecules that occur after injury and regenera-tion of the peripheral nerve. In the present study, the rat median nerve was transected, and transcriptional changes were studied at day 6 and day 12 after injury in both the proximal and the distal stumps, in the ab-sence or presence of microsurgical repair. The expres-sion profiles of the following genes were investigated: three metallothionein isoforms (MT-1, MT-2, and MT-3), the main antioxidant enzymes (catalase, superoxide dismutase, and glutathione-S-transferase), and the marker of cellular damage poly(ADP-ribose) polymer-ase-1 (PARP-1). The results showed that, in the proxi-mal nerve stump, MT-3 mRNA expression was signifi-cantly and markedly up-regulated in the absence of surgical repair, whereas MT-1 and MT-2 showed signifi-cant down-regulation. In the distal nerve portion, mRNA expression of all MT isoforms decreased significantly in the absence of microsurgical reconstruction, whereas, after repair, MT-3 mRNA expression alone was up-regu-lated. Expression of all the antioxidant enzymes decreased significantly after repair in the proximal nerve portion, but a significant general increase in their mRNA expression was revealed in the distal nerve stump. PARP-1 expression was significantly up-regu-lated in the proximal nerve portion without repair but dramatically reduced after reconstruction. In contrast, PARP-1 expression increased markedly in the distal stump after surgical repair. Taken together, these find-ings indicate that antioxidant molecules are differen-tially modulated and might, therefore, play an important role in peripheral nerve injury and regeneration.

Key words: peripheral nerve injury; regeneration; metallothionein expression; oxidative stress; rat

Although peripheral nerve injury is one of the most important and frequent types of lesion in industrial societies, the molecular mechanisms of nerve regeneration are still unclear, and a complete recovery of nerve function after repair is almost never achieved in spite of the intrinsic capacity of peripheral nerve fibres to regen-erate after axotomy (Evans, 2001; Siemionow et al., 2009; Geuna et al., 2009; Deumens et al., 2010; Oliveira et al., 2010). Previous studies have shown that peripheral nerve injury induces the production of reactive oxygen species (ROS) and nitric oxide (NO) in axotomized neurons (Bowe et al., 1989; Clarke and Richardson, 1994; Kubo et al., 2002; Zochodne and Levy, 2005). Moreover, Schwann cells (SCs) and macrophages in the injured nerve express proinflammatory molecules such as interleukin (IL)-1B, IL-6, IL-12, tumor necrosis factor-a (TNF-a), and transforming growth factor-b (TGF-b; Terenghi, 1999), and these in turn induce the transcrip-tion of several enzymes, including nitric oxide synthases (NOS; McDonald et al., 2007).

Cells and tissues are equipped with a variety of en-zymatic and nonenzymatic antioxidants to remove excess ROS and protect against oxidative injury (Hensley et al., 2000). Among the nonenzymatic systems, metallothio-neins (MTs) are the most important in the nervous sys-tem. MTs are a class of low-molecular-weight (6–7 kDa) metal-binding proteins involved in scavenging free radi-cals (Chung and West, 2004), storage and metabolism of essential metals, and detoxification of toxic metals (Ver-gani et al., 2007). In mammals, four major subfamilies have been identified (from MT-1 to MT-4). MT-1 and MT-2 are expressed almost ubiquitously, and in rodents they are often described as a unique functional entity (Penkowa, 2006). In the central nervous system (CNS), MT-1 and MT-2 exert neuroprotective effects by acting as a defense against heavy metals and oxidative stress (Hidalgo et al., 2001); they are expressed in largely astro-cytes and spinal glia and are almost absent from neurons. MT-3 is abundant in neurons and seems to be relevant to neuronal Zn^{21} homeostasis, even though the neuro-nal roles of this isoform are still unclear, and divergent data have been reported (Frazzini et al., 2006). A direct correlation has been described between the expression of MTs and the onset of several neurodegenerative diseases, including Parkinson's disease, multiple sclerosis, and amyotrophic lateral sclerosis, as well as their animal models (Penkowa and Hidalgo, 2003; Ebadi et al., 2005). MT-3 was originally called growth-inhibitory factor (GIF), because it was reported to inhibit neuron survival in vitro (Uchida et al., 1991). In contrast to that work, several in vitro results over the past few years have sup-ported a neuroprotective role for MT-3.

To the best of our knowledge the modulation of MTs after peripheral nerve injury has not yet been investigated, so the goal of this study was to analyze their expression profiles after complete transection, with or without subsequent microsurgical epineural repair, in the rat median nerve model at 6 and 12 days posttrauma. Expression of the main antioxidant enzymes, catalase, superoxide dismutase, and glutathione-S-transferase, was also

evaluated. Finally, we also assessed the expression of a typical marker of cellular damage and apoptosis, namely, poly(ADP-ribose) polymerase-1 (PARP-1).

MATERIALS AND METHODS

Chemicals

All chemicals, unless otherwise indicated, were of ana-lytical grade and were obtained from Sigma-Aldrich (Milan, Italy).

Animal Surgery

In total, 20 adult female Wistar rats (Charles River Lab-oratories, Milano, Italy) were used. Animal cages were housed in a temperature- and humidity-controlled room with 12/12-hr light/dark cycles. The animals were fed with standard chow and water ad libitum. Measures were taken to minimize pain and discomfort, taking into account human endpoints for animal suffering and distress. All procedures were performed with the approval of the local institution's Animal Care and Ethics Committee and in were accordance with the European Communities Council Directive of 24 November 1986 (86/ 609/EEC).

With animals under deep anesthesia, after trichotomy and under clean conditions, the median nerve of the left fore-limb was accessed from the axillary region to the elbow using a longitudinal skin approach. Under the operative microscope, the median nerve was carefully exposed from its origin in the brachial plexus to the elbow, then transected at the level of the pectoralis major muscle. In 10 animals, the nerve was im-mediately repaired in an end-to-end fashion by means of two epineurial stitches of 11-0 monofilament nylon. In another group of animals (n 5 10), the nerve was left unrepaired after withdrawal of a 2-mm-long segment to avoid spontaneous regrowth. Each of the two groups was then further divided into two groups of animals (n 5 5) that were euthanized, under deep anaesthesia, at postoperative day 6 or 12, and the nerves were approached again. From each nerve, 8-mm-long segments were withdrawn both proximal and distal to the lesion site. In the neurorrhaphy group, the anastomosis site was removed before the proximal and distal segments were collected. Each sample was then divided into two segments, one of which was fixed in 2.5% glutaraldehyde for histological analysis, whereas the other was frozen at 2808C for RT-PCR analysis.

Histology

For histological analysis, nerve samples after fixation in 2.5% glutaraldehyde were postfixed in 1% osmium tetroxide, dehydrated, and embedded in resin as described by Raimondo et al. (2009). From each nerve, serial 2-lm-thick semithin transverse sections were cut using an Ultracut UCT ultrami-crotome (Leica Microsystems, Wetzlar, Germany) and stained with toluidine blue for high-resolution light microscopy.

RNA Extraction and Real-Time RT-PCR

Total RNA was isolated by the acid phenol-chloroform procedure (Chomczynski and Sacchi, 1987) using Trizol rea-gent (Sigma) according to the manufacturers' instructions. The purity of the RNA was checked via absorption spectroscopy by measuring the 260/280 ratio. Only high-purity samples

($OD_{260/280}$ > 1.8) were subjected to further manipulation. The quality of the isolated RNA was assessed by electrophoresis on 1.5% formaldehyde-agarose gel to verify the integrity of the 18S and 28S rRNA bands. Single-strand cDNA was synthesized from 1 lg of total RNA from each sample using 200 ng oligo(dT)18-primer (TIB Mol Biol, Genoa, Italy), 200 U RevertAid H-Minus M-MuLV reverse transcriptase (Fer-mentas, Hannover, MD), 40 U RNAsin, and 1 mM dNTPs (Promega, Milan, Italy) in a final volume of 20 ll (Vergani et al., 2007). The reaction was performed in a Master Cycler apparatus (Eppendorf, Milan, Italy) at 428C for 1 hr after an initial denaturation step at 708C for 5 min.

The expression levels of the selected genes were quanti-fied in 96-well optical PCR plates using a Chromo 4 System real-time PCR apparatus (Bio-Rad, Milan, Italy). Real-time PCRs were performed in quadruplicate in a final volume of 20 ll containing 10 ng cDNA, 10 ll iTaq SYBR Green Supermix with ROX (Bio-Rad), and 0.25 lM of each primer pair (TibMolBiol). No cDNA was added to the negative con-trols. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene to normalize the expression data, as previously described (Lanza et al., 2009). The primer pairs for the genes under analysis are listed in Table I. [T1]

The thermal protocol included an initial denaturation step at 958C for 3 min, and 40 cycles of 958C (15 sec),

608C (30 sec), and 728C (20 sec). The melting curves of the PCR products (from 55 to 948C) were also recorded to check the reaction specificity. In each sample, the relative amount of a specific mRNA sequence was calculated by the comparative DDCt method (Pfaffl et al., 2002). This method involves first calculating the difference between the Ct values (DCt) of target and housekeeping genes, then the difference between the Ct val-ues of the sample and the calibrator (control). Therefore, the nor-malized expression was expressed as the relative quantity of mRNA (fold induction) with respect to the control sample. The Bio-Rad software tool Genex-Gene Expression Macro (Vande-sompele et al., 2002) was used for data elaboration. Data are the mean 6 SD of three experiments repeated in quadruplicate.

TABLE I. Names and Accession Numbers of the Target Genes are Listed Together With the Sequences of the Specific Primer Pairs (http://www.ncbi.nlm.nih.gov/pubmed/)

Gene name	Accession Number	Forward primer [5′-3′]	Reverse primer [5′-3′]
MT-1	NM_013602	CTGCTCCACCGGCGG	GCCCTGGGCACATTTGG
MT-2	NM_008630	TCCTGTGCCACAGATGGATC	GTCCGAAGCCTCTTTGCAGA
MT-3	NM_013630	GGAGGAACCAAGCTACGGC	ACATAGGCTGTGTGGGAGGG
PARP-1	NM_013063	TGCAGTCACCCATGTTCGATGG	AGAGGAGGCTAAAGCCCTTG
GST	NM_013541	GTGCCCGGCCCAAGAT	TTGATGGGACGGTTCACATG
CAT	NM_009804	CCTGAGAGAGTGGTACATGC	CACTGCAAACCCACGAGGG
GAPDH	NM_008084	GACCCCTTCATTGACCTCAAC	CGCTCCTGGAAGATGGTGATGGG

Statistical Analysis

Data on real-time RT-PCR and enzymatic activities are mean 6 SD of two independent RNA extractions from two in-dependent experiments performed in quadruplicate. Statistical analysis was performed by ANOVA, followed by the Bonferroni ad hoc posttest (INSTAT; GraphPad Software, San Diego, CA).

RESULTS

Histology

The results of the histological analysis, upstream and downstream of the lesion site, are shown in Figure 1. At postoperative day 6, the proximal stumps show normal nerve morphology in both repaired and unre-paired nerves with large- and small-gauge nerve fibres with myelin sheaths of proportional thickness. In the dis-tal stumps, axons with myelin degeneration (Wallerian degeneration) can be detected in both groups. At post-operative day 12, the proximal stumps maintain normal nerve morphology, whereas, in the distal stumps, together with ongoing Wallerian degeneration, the reconstructed nerves show denser interstices. Although myelination has still not begun at this postoperative stage of regeneration, so that axons are not detectable by light microcopy, the rich interstices could be interpreted as an indirect sign of initial axonal regeneration.

Expression of MTs During Nerve Regeneration

The expression patterns of the three MT isoforms were assessed by real-time RT-PCR in both the distal and the proximal nerve portions at two different times (6 and 12 days) after nerve transection in the presence or absence of microsurgical repair. The expression level of each MT isoform in samples under different postopera-tive conditions was normalized with respect to the con-trol (nerve without transection). Transcripts of metallothioneins MT1 and -2 were already constitutively present in control specimens from untreated animals.

In the proximal nerve portion without surgical repair (DP), a significant and marked increase in mRNA expression of MT-3 was observed at both day 6 and day 12 with respect to controls (1.4- and 1.5-fold for DP_6 and DP_12, respectively; $P < 0.001$; Fig. 2A). In contrast, MT-3 mRNA expression showed a significant and marked decrease when the proximal region was sutured (TTP) to the distal portion (0.03- and 0.28-fold for TTP_6 and TTP_12, respectively; $P < 0.001$; Fig. 2A).

Expression of the MT-1 and MT-2 isoforms decreased significantly at all times under all conditions in the proximal nerve portion (Fig. 2A). In the absence of microsurgical repair, expression of MT-1 and MT-2 decreased markedly with respect to control (0.65- and 0.58-fold, respectively; $P < 0.01$) at day 6 (DP_6) as well as at day 12 (DP_12; 0.45-fold for MT-1 and 0.78- fold for MT-2 with respect to control; $P < 0.001$ and $P < 0.05$, respectively). After microsurgical repair at day 6 (TTP_6), the expression of MT-1 and MT-2 decreased significantly with respect to control (0.25-fold for MT-1 and 0.50 fold for MT-2; $P < 0.001$ and $P < 0.01$, respectively), but, at day 12 (TTP_12), the expression rose toward values close to control (0.71- and 0.72-fold for MT-1 and MT-2, respectively, with respect to control; $P < 0.01$).

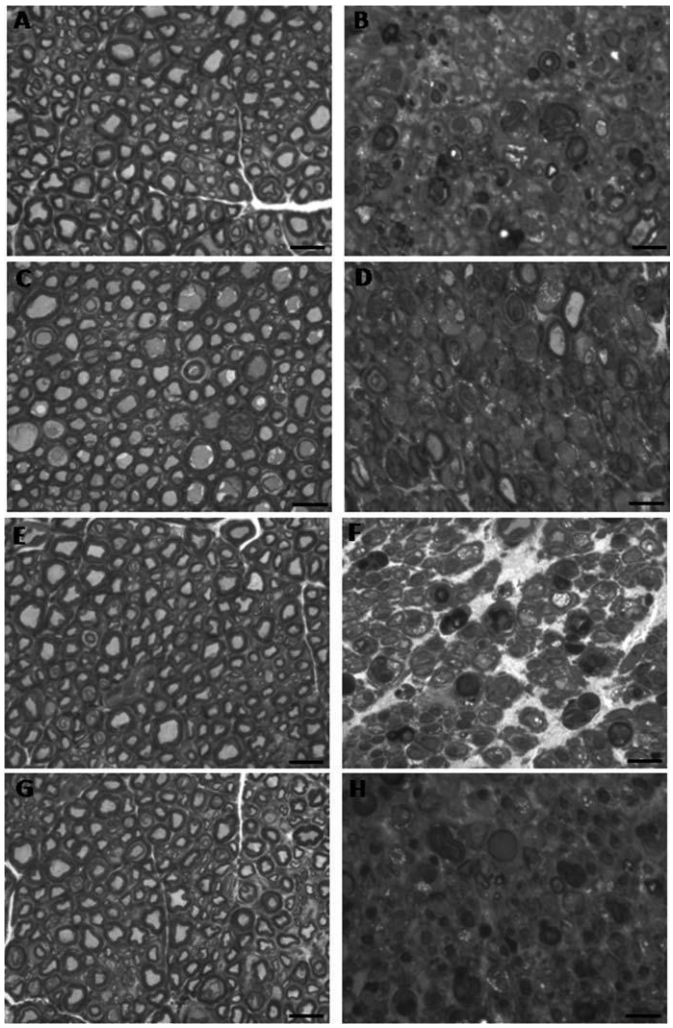

Fig. 1. Histology. A–D: Postoperative day 6. Photomicrographs of semithin sections cut transversely to the main axis of proximal (A) and distal (B) stumps of the median nerve not followed by micro-surgical suture, and of proximal (C) and distal (D) stumps of the median nerve after end-to-end repair. E–H: Postoperative day 12. Photomicrographs of semithin sections cut transversely to the main axis of proximal (E) and distal (F) stumps of the median nerve not followed by microsurgical suture and of proximal (G) and distal (H) stumps of the median nerve after end-to-end repair. Scale bars 5 10 lm.

In the distal nerve portion, expression of MT-3 showed changes opposite to those observed in the proximal portion (Fig. 2B). In the absence of suture (DD), the MT-3 mRNA level showed a dramatic decrease at day 6 (DD_6) that was only slightly reversed at day 12 (DD_12) with respect to control (0.06- and 0.3-fold, respectively; $P < 0.001$). In contrast, when the proximal region was sutured to the distal portion (TTD), MT-3 expression increased significantly with respect to control (2.19- and 3.0-fold in TTD_6 and TTD_12, respectively; $P < 0.001$).

In the distal nerve portion in the absence of suture (DD), MT-1 mRNA expression was significantly downregulated (Fig. 2B) at both day 6 (DD_6) and day 12 (DD_12) with respect to control (0.58- and 0.60-fold, respectively; $P < 0.01$). MT-2 expression did not change significantly at day 6 (DD_6) and had decreased slightly at day 12 (DD_12; 1.19-fold with respect to control; $P < 0.001$; Fig. 2B). After microsurgical repair, expression of MT-1 and MT-2 decreased significantly at day 6 (TTP_6; 0.25- and 0.50-fold with respect to control; $P < 0.001$ and $P < 0.01$, respectively), but, at day 12 (TTP_12), the decrease in mRNA expression was significantly reduced (0.71- and 0.72-fold, respectively, with respect to control; $P < 0.01$; Fig. 2B).

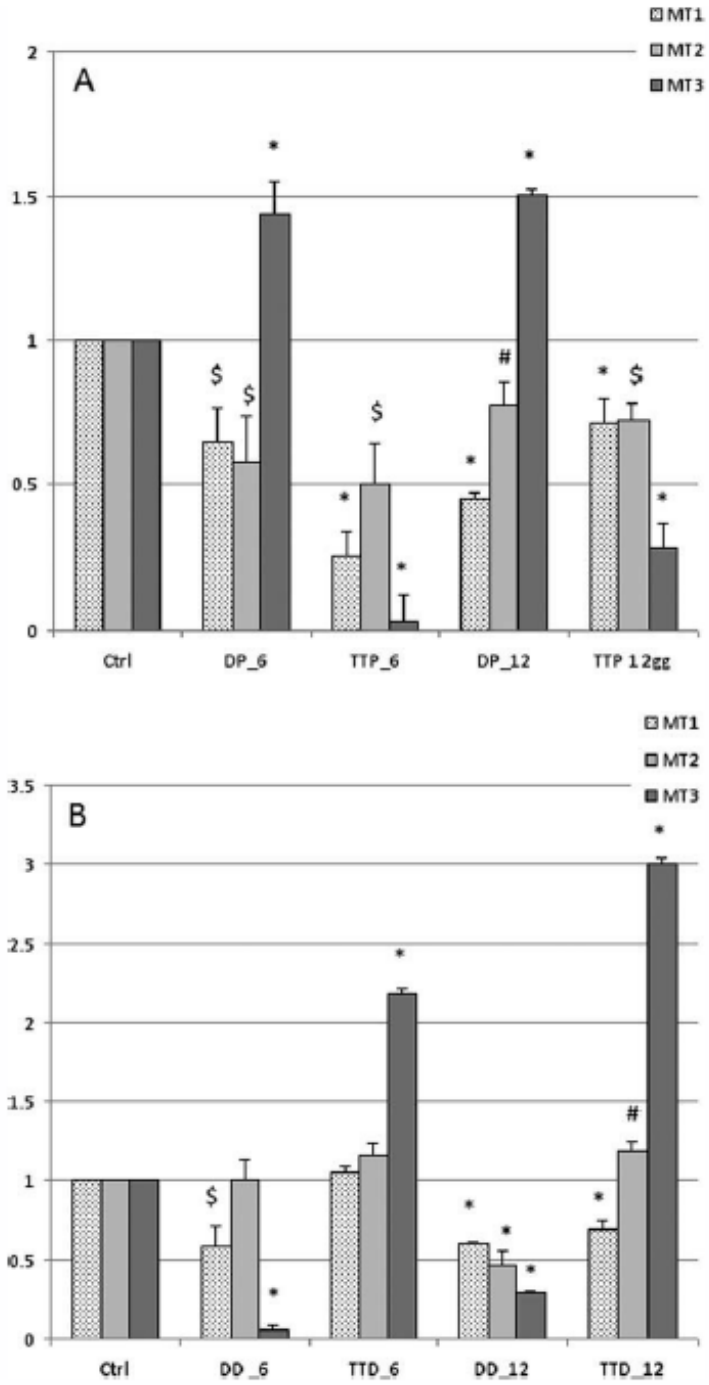

Fig. 2. Expression of metallothionein isoforms during nerve regeneration. Relative expression of three MT isoforms was quantified in both proximal (A) and distal (B) stumps of the median nerve after transection with or without suture. The mRNA levels of MT-1, MT-2, and MT-3 were quantified at days 0, 6, and 12 by real-time RT-PCR using GAPDH mRNA as a reference. Relative expression data (mean 6 SD) of four replicates are reported as fold induction with respect to controls. Differences among means were assessed by ANOVA, followed by the Bonferroni ad hoc posttest, and are denoted by symbols on the bars. *Groups that are significantly different from control ($P < 0.001$). $Groups that are significantly different from control ($P < 0.01$). #groups that are significantly different from control ($P < 0.05$).

Expression of Antioxidant Enzymes During Nerve Regeneration

The transcript levels of the main antioxidant enzymes involved in oxyradical detoxification were also evaluated: catalase (CAT) and Cu,Zn-superoxide dismutase (SOD), as well as glutathione transferase (GST), which is involved in detoxification of various substrates through consumption of the main soluble cellular thiol, glutathione (GSH). As shown in Figure 3A, no signifi-cant changes in the mRNA expression of the three anti oxidant enzymes were observed in the proximal nerve portion without surgical repair at day 6 (DP_6), whereas, at day 12 (DP_12), there was a significant decrease only in CAT expression (0.56-fold with respect to control; $P < 0.001$). There were no significant changes in GST expression at any time. When the prox-imal region was sutured to the distal portion, the mRNA expression of all three enzymes was significantly downregulated (Fig. 3A). At day 6 (TTP_6), expression of GST, CAT, and SOD decreased markedly with respect to control (0.56-, 0.26-, and 0.53-fold, respec-tively; $P < 0.01$), but, at day 12 (TTP_12), GST and SOD expression rose slightly toward the control values (0.78- and 0.74-fold, respectively; $P < 0.05$), whereas CAT expression remained very low (0.30-fold with respect to control; $P < 0.001$; Fig. 3A).

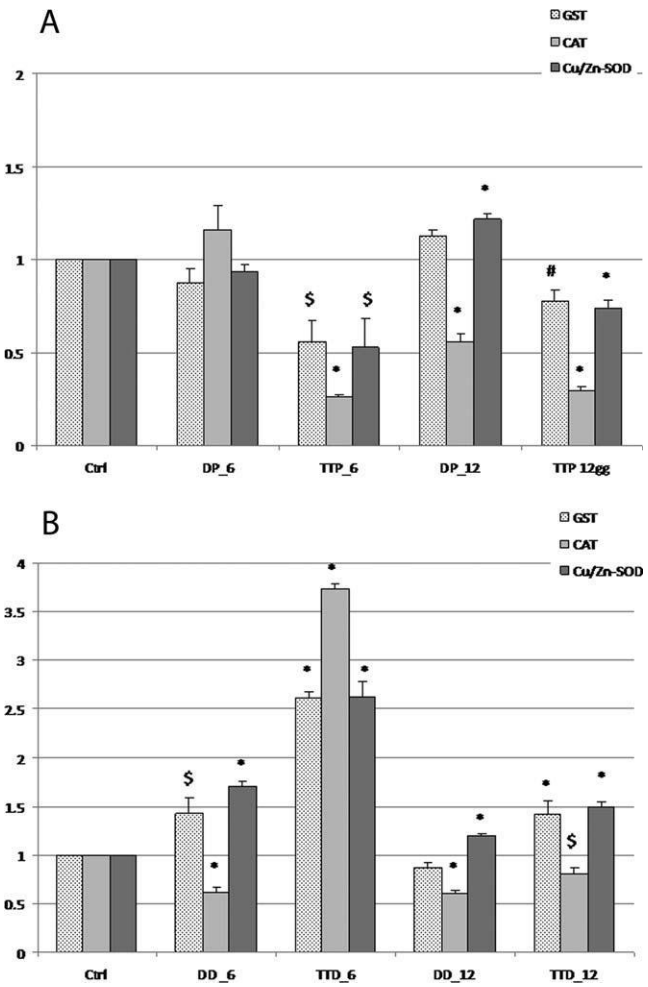

Fig. 3. Expression of antioxidant enzymes during nerve regeneration. Relative expression of catalase (CAT), Cu,Zn-superoxide dismutase (SOD), and glutathione transferase (GST) was quantified in both proximal (A) and distal (B) stumps of the median nerve after transec-tion followed or not by microsurgical suture at days 0, 6, and 12. *Groups that are significantly different from control ($P < 0.001$). $^{\$}$Groups that are significantly different from control ($P < 0.01$). $^{\#}$Groups that are significantly different from control ($P < 0.05$)

In the distal nerve portion without surgical repair at day 6 (DD_6), expression of GST and SOD increased significantly with respect to control (1.43- and 1.71-fold; $P < 0.01$ and $P < 0.001$, respectively), but CAT expres-sion was significantly downregulated (0.62-fold with respect to control; $P < 0.001$). At day 12 (DD_12), mRNA expression of GST and SOD had decreased. GST and CAT expression returned to control values, but SOD expression was

reduced with respect to control (0.60-fold; $P < 0.001$). When the proximal region was sutured to the distal (TTD_6), expression of GST and SOD was markedly up-regulated at day 6 with respect to control (1.42- and 1.50-fold, respectively; $P < 0.001$), but CAT expression was still rather low (0.80-fold with respect to control; $P < 0.001$; Fig. 3B).

Expression of PARP-1 During Nerve Regeneration

PARP-1 is a nuclear enzyme that contributes to both neuronal death and neuronal survival under stress conditions. In the proximal nerve portion without surgi-cal repair at day 6 (DP_6), PARP-1 expression was up-regulated with respect to control (1.69-fold; $P < 0.001$), and there was a further increase at day 12 (DP_12; 2.12-fold with respect to control; $P < 0.001$; Fig. 4A). An opposite trend was observed when the proximal region was sutured to the distal portion (TTP). At day 6 (TTP_6), PARP-1 mRNA expression was markedly downregulated (0.15-fold with respect to control; $P < 0.001$) and further decreased at day 12 (TTP_12; 0.075-fold with respect to control; $P < 0.001$; Fig. 4A).

In the distal nerve portion without surgical repair, mRNA expression of PARP-1 had decreased at day 12 (DD_12) with respect to control (0.33-fold, respectively; $P < 0.001$), whereas a marked increase occurred at day 6 after suture (TTD_6) with respect to control (5.69-fold; $P < 0.001$). This had decreased slightly again at day 12 (TTD_12; 2.46-fold with respect to control; $P < 0.001$; Fig. 4B).

DISCUSSION

The molecular mechanisms related to peripheral nerve injury and regeneration are still far from well understood despite the identification of numerous mole-cules involved in these processes. Elucidating the nature of these signals is a major goal for improving peripheral nerve repair (Dahlin et al., 2009; Fleming et al., 2009). Although oxidative stress is considered to be one of the main causes of neural damage (Martin et al., 1999, 2003), the expression of antioxidant proteins after nerve injury and repair has not been investigated so far to our knowledge.

To fill this gap partially, the present study was set up to investigate activation of the antioxidant system at days 6 and 12 after nerve injury. The rationale for the selection of these two time points deserves particular dis-cussion. The progress of axon regeneration is peculiar, in that it is related not only to the time passed after injury but also to the site along the distal nerve length (Geuna et al., 2009). Therefore, even if axonal regeneration begins at the injury site only a few hours after nerve reconstruction, its progression along the distal stump will take longer (1–3 mm/day). For this reason, we decided to select postoperative day 6 as the earlier time point to be sure that axon regeneration had already occurred all along the 8-mm-long distal nerve segment withdrawn for analysis. The same applies to the second time point selected (day 12), which should correspond to a more advanced stage of regeneration all along the 8-mm-long nerve segment.

The major findings were that 1) MT-3 mRNA expression was markedly up-regulated in the proximal nerve portion without surgical repair, whereas MT-1 and MT-2 mRNAs showed a general downregulation of their expression after injury; 2) MT-3 mRNA expression was upregulated in the distal nerve portion after surgical repair, whereas the mRNA expression of all MT iso-forms decreased in the absence of microsurgical repair; 3) in the proximal nerve portion, mRNA expression of all the antioxidant enzymes decreased after surgical repair; 4) in the distal portion, the antioxidant enzymes showed a general increase in their mRNA expression, with maximal upregulation at day 6 after surgical repair; and 5) PARP-1 expression was upregulated in the proximal nerve portion without surgical repair but was dramatically reduced after nerve surgery, whereas the oppo-site occurred in the distal stump.

In addition to the first comprehensive illustration of the changes in antioxidant molecules after nerve injury and repair, the patterns of MT-1, MT-2, and MT-3 transcription under the different experimental conditions are particularly interesting. In contrast to MT-1 and MT-2, which are preferentially expressed in nonneural cells such as astrocytes and Schwann cells, MT-3 is the neuron-specific metallothionein. A previous publication reported a significant role for MT-3, but not for MT-1 or -2, at the level of the regenerative process subsequent to nerve crush lesion (Ceballos et al., 2003). The present study shows that the expression of MT-1 and MT-2 was generally downregulated following nerve injury, indicating that these two antioxidant proteins have no significant role in the degenerative/regenerative processes in damaged nerves, in line with previous reports obtained on Mt1 1 -2KO and Mt3KO mice (Ceballos et al., 2003).

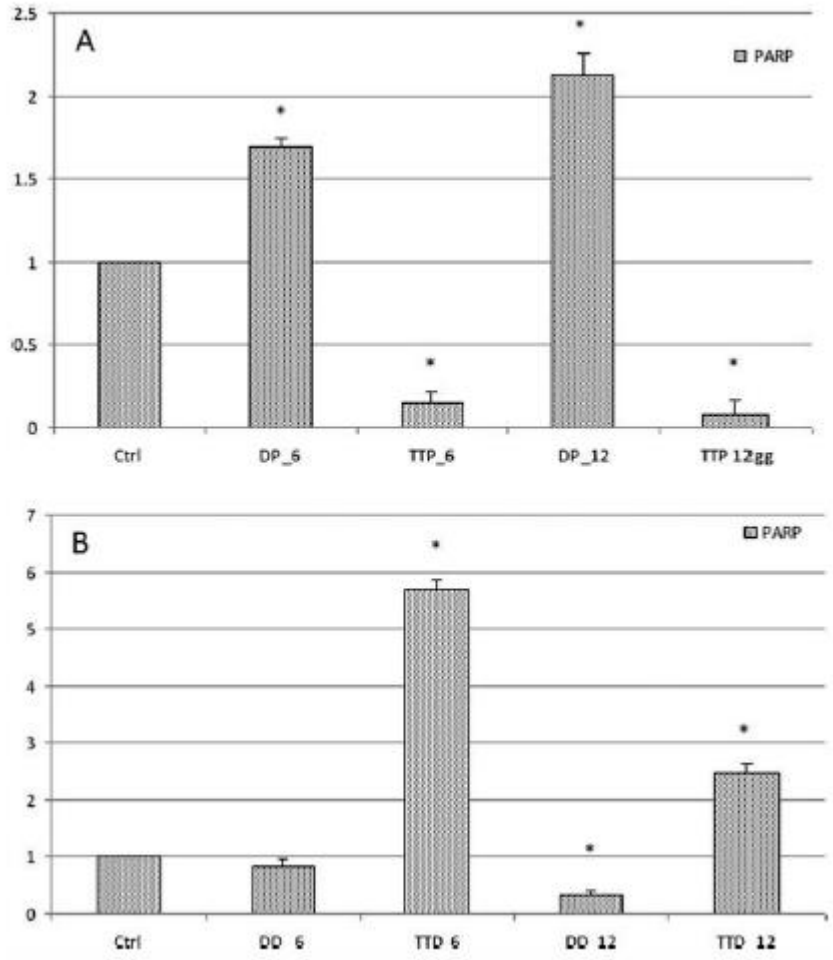

Fig. 4. Expression of PARP-1 during nerve regeneration. Relative expression of PARP-1 was quantified in both proximal (A) and distal (B) stumps of the median nerve after transection followed or not by microsurgical suture at days 0, 6, and 12. *Groups that are signifi-cantly different from control ($P < 0.001$).

On the other hand, our data show that the level of MT-3 mRNA increased significantly after nerve injury; this increase was localized in the proximal stump when there was no surgical repair, but in the distal stump after surgery. These results therefore suggest that increased levels of MT-3 are required in the nerve regions where posttraumatic regeneration processes take place. In fact, in the absence of surgical repair, high levels of MT-3 mRNA are expressed in the proximal stump, whereas, after surgical repair, MT-3 mRNA overexpression is de-tectable mainly in the distal nerve stump. The upregula-tion of MT-3 following nerve injury supports the view that this metallothionein exerts a neuroprotective role in vivo (Hozumi et al., 1995).

In summary, our results showed that, after periph-eral nerve injury, the antioxidant enzymes are upregu-lated in the distal stump, pointing to increased oxidative stress at this site. Moreover, our data showing maximal upregulation of enzyme expression after surgical repair indicate that activation of the antioxidant defense systems is specifically required in those regions involved mainly in the regenerative process (Ho¨ke, 2006). Finally, the results of the present study taken together point to a methodological approach that could be valuable for increasing our knowledge of the molecular mechanisms involved in peripheral nerve regeneration and, from the perspective of clinical application, might be useful for determining new therapeutic approaches targeted to antioxidant molecules.

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