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## Investigation of Peroxiredoxin IV as a Calpain-regulated Pathway in Cancer

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**Abstract.** *Peroxiredoxin IV (Prx IV), a member of the peroxiredoxin family, has been shown to be involved in cell protection against radiation. Peroxiredoxins are also overexpressed and involved in the progression of several tumours. Calpains have been shown to be over-activated in alveolar rhabdomyosarcoma (ARMS). The present study focused on the possible cross-regulations between Prx IV and calpains in ARMS cells. Prx IV abundance was quantified by Western blot analysis in ARMS cells and compared with non-malignant LHCN-M2 cells. Its abundance is quantified in ARMS cells treated or untreated with calpain inhibitors moreover its mRNA expression is also quantified by real-time RT-PCR in these cells. The study showed that Prx IV is overexpressed by five times in ARMS cells when compared to non-malignant myoblasts. Moreover, the inhibition of calpains using chemical inhibitors led to a decrease in Prx IV abundance ( $64.32 \pm 8.25$  and  $76.79 \pm 4.60$  for the precursor and secreted forms, respectively, with calpain inhibitor III treatment). It is the first time that a Prx IV calpain-dependent up-regulation is revealed. In summary, calpains may be implied in the tumour phenotype of ARMS cells especially through Prx IV regulation and may, thus, represent a potential therapeutic target to stop progression of ARMS tumour.*

A rhabdomyosarcoma (RMS) is a soft-tissue sarcoma commonly encountered in childhood and adolescence. RMS cells may acquire invasive behaviour and form metastases responsible for decreasing patient healing to

less than 20% (1). In spite of aggressive treatment strategies (surgery, chemotherapy and radiotherapy), this low rate of recovery underlines the necessity to understand the diverse mechanisms and pathways implied in alveolar rhabdomyosarcoma (ARMS) tumour aggressive phenotype. Such understanding may be a key for the development of new therapies to limit the generation and progression of this tumour.

Peroxiredoxins (Prx) have recently been the subject of growing interest as a new family of multifunctional thiol-specific antioxidant proteins (2). There are six isoforms of peroxiredoxin in mammalian cells, which can be divided into two groups, based on conservative cysteine residues: one group in which two cysteine residues are conserved (Prx I-IV), and the other group in which only one cysteine residue is conserved (Prx V, VI) (3).

Prx-IV was recently isolated and is highly homologous to other Prx members. It has a distinct hydrophobic NH<sub>2</sub>-terminal sequence that appears to be a signal sequence responsible for secretion outside the cell (4). Prx IV has been shown to serve as a regulatory factor for NF- $\kappa$ B, an oxidative stress-activated transcriptional factor responsible for the activation of cell-survival genes. Moreover, this peroxidase may play a role in protecting cells from radiation-induced apoptosis in head-and-neck squamous cell carcinoma (5). In lung carcinoma, Prx I and IV levels are significantly elevated compared to non-malignant tissue and high Prx IV expression is associated with high-grade squamous cell carcinoma (6). In the same way, the overexpression of Prx IV was described in osteosarcoma (7). This overexpression is also present in breast carcinoma (8), where PRX IV is involved in tumour development and progression (9). Dysregulation of oxygen influx in the tumour microenvironment may have a potential role in carcinoma angiogenesis and invasiveness. Prx IV presents two forms, namely the 31 kDa endoplasmic precursor form and the secreted 27 kDa form (10-11). The secreted form may be involved in the regulation of the interactions between carcinoma cells and their environment.

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Several studies indicate that Prx may be cleaved and their activity modulated by calpains, calcium-dependent endoproteases. The cleavage of Prx III by calpains induces a decrease of peroxidase activity (12). Calpains may also contribute to the degradation of Prx I and II since an ionophore treatment induced a decrease in the abundance of both peroxidases (13). However, the degradation of Prx I by calpain may be less efficient than that of Prx II.

The best characterised calpains are  $\mu$ -calpain and m-calpain. Both are calcium-dependent endoproteases presenting a cystein-histidin-asparagin catalytic triad. They are heterodimers encoded by *capn1* and *capn2*, for the  $\mu$ - and m-catalytic subunit (80 kDa), respectively (14-16) and by the *css1* and *css2* genes, for the small regulating subunit (30 kDa). Calpain activity is  $\text{Ca}^{2+}$ -dependent and is mainly regulated by calpastatin, its specific endogenous inhibitor. The deregulation of calpains has been involved in tumour invasion and metastasis in several different cell types. Specifically, their overactivity has been shown in ARMS and a strong positive linear correlation has been made between calpain activity and migration velocity. Calpains may, thus, be considered as markers of tumour aggressiveness (17).

Since the implication of Prx IV and calpains in several cancers and because there is an already well-established link between Prx I, Prx II and calpains, the present study focused on the possible interactions between Prx IV and calpains in ARMS cells. Prx IV abundance was first determined in ARMS cells and compared to that of non-tumour human myoblasts (LHCN-M2). Then, a calpain-dependent potential regulation of Prx IV was evaluated both at the protein and at the mRNA level of Prx IV by Western blot analysis and by real-time RT-PCR. The main results indicated that Prx IV is overexpressed in ARMS cells at the protein level and this up-regulation may be due to a calpain-regulated pathway.

## Materials and Methods

**Cell culture.** In order for the cells to expand, they were grown under 5%  $\text{CO}_2$  atmosphere at 37°C in DMEM (Dulbecco's modified Eagle medium, Invitrogen, Cergy Pontoise, France) with pyruvate supplemented with 10% foetal bovine serum (FBS, Invitrogen, Cergy Pontoise, France) for ARMS, and a culture medium containing DMEM without pyruvate (60%), medium 199 (Invitrogen, Cergy Pontoise, France) (20%) and FBS (20%) for LHCN-M2 cells (18). For all experiments, the cells were placed for 48 h in a quiescent medium (DMEM supplemented with 0.1% FBS) in condition of migration, which is a key step for metastatic dissemination.

**Calpain inhibitor treatment.** The chemical calpain inhibitors, Calpeptin (Z-Leu-Nle-CHO) and calpain inhibitor III (Z-Val-Phe-CHO) were obtained from VWR (Fontenay-sous-bois, France) and used at concentrations of 100  $\mu\text{M}$  and 50  $\mu\text{M}$ , respectively. Inhibitors of calpains were added 4 h before extraction of proteins or total RNA.

**Western blots.** Western blots were performed as described previously (17). The bicinchoninic acid protein assay kit was obtained from Pierce Science (Brenbère, France), the polyvinylidene fluoride membrane from Millipore Corporation (Billerica, USA) and nitroblue tetrazolium/5-bromo-4-chloro-3-indoyl-1-phosphate from Promega (Charbonnières-les-Bains, France). The membranes were incubated with antibodies against Prx IV (anti-PRX IV goat IgG, Santa Cruz Biotechnology, California, USA, (sc-23974); 1/500 or 1/200 for Western blot ARMS and LHCN-M2). Secondary conjugated alkaline phosphatase antibodies (Sigma-Aldrich, Saint Quentin Fallavier, France, (A4187); 1/10000) were used for detection. The apparent density of the band on the membranes was quantified with Totalab (Nonlinear Dynamics, Newcastle Upon Tyne, UK).

**Extraction of total RNA, reverse transcription and quantitative PCR.** Extraction of total RNA, reverse transcription and quantitative PCR were performed as previously described (17). Forward and reverse primer sequences for Prx IV amplification were PIFb (tgacctaagcaagcgag) and P2Ra (gaatcaacagagcatgctacc), respectively (19). The Prx IV transcript concentration of ARMS treated with calpain inhibitor III was determined from the standard curve, established from control-cell genomic ADN and compared to non-treated ARMS.

**Statistical analysis.** The statistical significance of the differences between multiple groups was determined using analysis of variance. When the Fisher's *F* statistic was associated with a probability  $p < 0.05$ , intergroup comparisons were conducted using the Student's *t*-test. Unless otherwise indicated, all data were expressed as mean  $\pm$  standard error of the mean (SEM), calculated from at least three independent experiments.

## Results and Discussion

**Prx IV is overexpressed in RMS cells.** Prx IV has been shown to be overexpressed in some human carcinomas but nothing was known about its expression in RMS cells. In the present study, the Prx IV expression was quantified by Western blot analysis in ARMS cells and compared to that of non-malignant LHCN-M2 cells (Figure 1). In the ARMS cells, Prx IV was significantly overexpressed by five times compared to the LHCN-M2 myoblasts ( $486.65 \pm 4.40\%$ ). This Prx IV overexpression was in agreement with the high levels of Prx IV in breast, lung, head-and-neck carcinoma and osteosarcoma (5-8). Since Prx IV is involved in the detoxification of reactive oxygen species (20), the proliferation and development of breast tumour cells (9) and the protection from radiation-induced apoptosis in head-and-neck squamous carcinoma (5), the overexpression of Prx IV may be partly responsible for the high aggressiveness of the ARMS tumour.

**Expression of Prx IV is a calpain-dependent mechanism.** It has been shown in rat cells that Prx I and Prx II may be cleaved by calpains (13). Furthermore, ARMS cells have shown a high calpain activity (17). In spite of these data, a possible regulation of Prx IV by calpains was still never

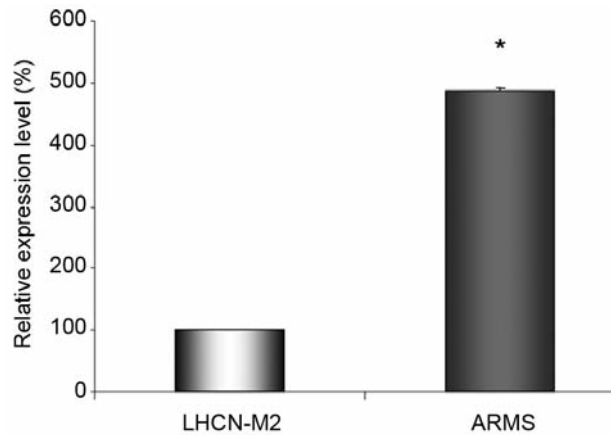


Figure 1. Relative abundance of Prx IV in ARMS compared to LHCN-M2. Cells were placed for 48 h in a quiescent medium, under migration condition. Proteins from ARMS and LHCN-M2 were extracted and subjected to SDS-PAGE. Immunodetection was performed using specific antibodies for Prx IV. Results represent mean $\pm$ SEM from at least three independent experiments. \*Significantly different from the control LHCN-M2 ( $p < 0.05$ ).

evoked. To achieve this in the present study, the Prx IV expression was quantified in ARMS cells, by Western blot analysis, in the presence or absence of calpain inhibitors.

Calpain inhibition by calpain inhibitor III led to a significant decrease of the precursor and the secretable forms ( $64.32 \pm 8.25$  and  $76.79 \pm 4.60$ , respectively) in comparison with non-treated cells (Figure 2A). Similar results were obtained when calpains were inhibited by calpeptin ( $52.17 \pm 5.00$  and  $86.12 \pm 3.15$  for the precursor and secretable forms, respectively) (Figure 2B).

These results indicated that, contrary to osteosarcoma Prx I and II (7), in ARMS cells, Prx IV would not be a calpain substrate. However, these data showed that Prx IV is up-regulated by calpains, suggesting, for the first time, that the proteases are involved in a peroxidase regulation pathway.

*The regulation of Prx IV by calpain takes place at the post-transcriptional level.* To specify the mode of calpain-mediated Prx IV regulation, Prx IV-specific mRNA levels were quantified by real-time RT-PCR in ARMS cells treated with calpain inhibitor III and compared to that of non-treated ARMS cells (Figure 3).

Prx IV mRNA level was similar in ARMS treated with calpain inhibitor III and in non-treated ARMS cells. However, Western blot analysis showed that Prx IV was significantly reduced in calpain-inhibited ARMS cells when compared to the control cells. These results may be explained by a potential post-transcriptional regulation of ARMS Prx IV by calpains.

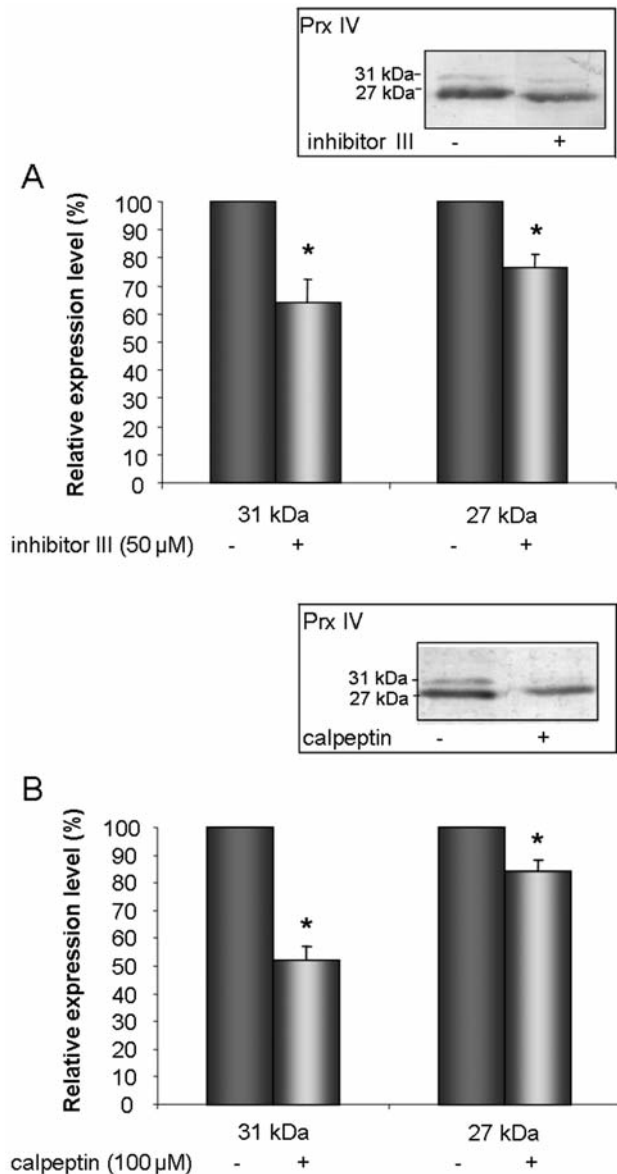


Figure 2. Relative abundance of Prx IV in ARMS treated (+) or untreated (–) with (A) calpain inhibitor III (50  $\mu$ M) or (B) calpeptin (100  $\mu$ M). Cells were placed for 48 h in a quiescent medium, in migration condition. Inhibitor III or calpeptin were added to cell medium 4 h before protein extraction. Proteins from ARMS treated or untreated with calpain inhibitor were extracted and subjected to SDS-PAGE. Immunodetection was performed using specific antibodies for Prx IV. Results represent mean $\pm$ SEM from at least three independent experiments. \*Significantly different from the untreated control ( $p < 0.05$ ).

It is the first time that such a regulation has been revealed. Taken together, these data demonstrate that calpains up-regulate Prx IV and that this stimulation is independent of regulation at mRNA level.

These data, however, disagreed with the data obtained by Lee *et al.* (9) who observed an increase in both protein and

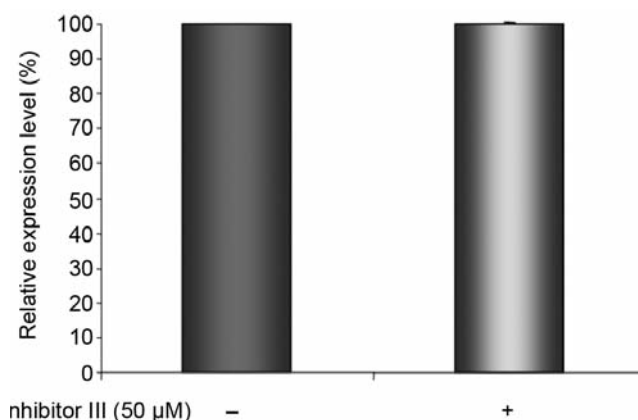


Figure 3. Quantification of Prx IV mRNA expression in ARMS treated or untreated with calpain inhibitor III (50  $\mu$ M). Quantification of Prx IV mRNA expression was performed by real-time RT-PCR. Forward and reverse primer sequences for Prx IV amplification were Pifb (tgacctaagcaagcgaag) and P2Ra (gaatcaacagagcatgtacc), respectively. Results represent the mean $\pm$ SEM from at least three independent experiments.

mRNA levels in MCF-7 cells, leading to a stimulation of cancer development and progression. Such an increase in mRNA of Prx IV was also shown in lung cancer (6).

These results showed that the expression of Prx IV is increased at the protein level in ARMS cells. Moreover, the inhibition of calpains led to a decrease in this overexpression. Therefore, in ARMS, Prx IV is not a calpain substrate. Furthermore, this is the first time that a calpain-mediated up-regulation of Prx IV is demonstrated. Several hypotheses may be envisaged to explain this regulation of Prx IV by calpains. Calpains may be involved in the up-regulation of chaperone proteins, thus, increasing the protein stability of Prx IV. Calpains may also intervene in the down-regulation of Prx IV degradation pathway by preventing, for example, its ubiquitination and, thus, its degradation by the proteasome system.

In breast cancer, Lee *et al.* (9) showed that Prx IV overexpression may stimulate the proliferation and be involved in cancer development and progression. In the same way, in ARMS cells, calpains may be involved in the stimulation of cell proliferation, tumour development and progression by the up-regulation of Prx IV. Furthermore, in head-and-neck cell carcinoma, it was shown that Prx IV would protect cells from radiation-induced apoptosis (5). In ARMS cells, calpains may be partially responsible for this phenomenon.

In summary, calpains may be implicated in the tumour phenotype of ARMS cells by stimulating their metastatic dissemination (17) but also in their proliferation and their resistance to the radiation by the up-regulation of Prx IV. For

these reasons, calpains may represent a potential therapeutic target to stop the metastatic progression of ARMS tumour and, thus, increase patient healing rates.

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