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Increased poly(ADP-ribose)ylation in skeletal muscle tissue of pediatric patients with severe burn injury: prevention by propranolol treatment

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Summary

Activation of the nuclear enzyme poly (ADP-ribose) polymerase (PARP) has been shown to promote cellular energetic collapse and cellular necrosis in various forms of critical illness. Most of the evidence implicating the PARP pathway in disease processes is derived from preclinical studies. With respect to PARP and burns, studies in rodent and large animal models of burn injury have demonstrated the activation of PARP in various tissues and the beneficial effect of its pharmacological inhibition. The aim of the current study was to measure the activation of PARP in human skeletal muscle biopsies at various stages of severe pediatric burn injury and to identify the cell types where this activation may occur. Another aim of the study was to test the effect of propranolol (an effective treatment of patients with burns), on the activation of PARP in skeletal muscle biopsies. PARP activation was measured by Western blotting for its product, poly(ADP-ribose) (PAR). The localization of PARP activation was determined by PAR immunohistochemistry. The results showed that PARP becomes activated in the skeletal muscle tissue after burns, with the peak of the activation occurring in the middle stage of the disease (13–18 days after burns). Even at the late stage of the disease (69–369 days post-burn) an elevated degree of PARP activation persisted in some of the patients. Immunohistochemical studies localized the staining of PAR primarily to vascular endothelial cells, and occasionally to resident mononuclear cells. There was a marked suppression of PARP activation in the skeletal muscle biopsies of patients who received propranolol treatment. We conclude that human burn injury is associated with the activation of PARP. We hypothesize that this response may contribute to the inflammatory responses and cell dysfunction in burns. Some of the clinical benefit of propranolol in burns may be related to its inhibitory effect on PARP activation.

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Keywords

nitric oxide; superoxide; burns; inflammation; contraction; vascular; poly (ADP-ribose) polymerase; endothelial cell; peroxynitrite

Introduction

Overproduction of various oxygen- and nitrogen-derived reactive species is a common feature of various forms of critical illness. The reactive oxygen species (superoxide, hydrogen peroxide, hydroxyl radical) are produced by multiple sources, such as NADPH oxidases, catechol and prostanoid auto-oxidation and mitochondrial respiratory enzymes. The reactive nitrogen species nitric oxide ($\cdot\text{NO}$) is produced constitutively at low levels by many cells; in diseases it is produced at higher levels upon the increased expression of its inducible isoform (iNOS). In many pathophysiological conditions, NO and superoxide ($\text{O}_2^{\cdot-}$) react to form the toxic reaction product, peroxynitrite anion (ONOO^-) (reviewed in 1). Reactive oxygen species, on their own, or after conversion into peroxynitrite, can mediate a multitude of deleterious actions in critical illness. These actions (reviewed in 1,2) include lipid peroxidation reactions, protein oxidation, DNA strand breakage and DNA base modification, and depletion/inhibition of cellular antioxidants. The end-results of these processes in critical illness include inhibition of cell respiration, cell injury and cell death (apoptosis, necrosis) as well as immune/inflammatory dysfunction.

The nuclear enzyme PARP-1 is a highly conserved 116 kDa protein, which is present in most mammalian cells. Upon binding to damaged DNA, PARP-1 becomes activated and catalyzes the cleavage of NAD^+ into nicotinamide and ADP-ribose to form long branches of ADP-ribose polymers (PAR) on nuclear proteins including PARP-1 itself. Poly(ADP-ribosylation) is a process involved in DNA repair and transcriptional regulation. PARP activation can lead to marked changes in cell energetics; it can promote cell death; and it can also affect immune/inflammatory mediator production. PARP activation occurs in many forms of critical illness, due to reactive oxidant formation and consequent DNA injury. Inhibition or genetic inactivation of PARP improves outcome in various animal models of critical illness (reviewed in 2–4).

Preclinical studies have implicated the peroxynitrite/PARP pathway in the pathogenesis of burn injury. Peroxynitrite is a key trigger of PARP activation, because (1) it is readily formed during oxidative stress; (2) it has a relatively long half-life, which enables it to travel from one cell to another by crossing cell membranes and (3) it induces DNA single strand breaks, which triggers PARP activation (1). Formation of peroxynitrite and PARP activation are simultaneously present in many human diseases (2). Several series of studies suggest that the PARP pathway may play a role in the pathophysiology of burn injury. First, peroxynitrite is formed in rodent models of burn injury, both locally (burned skin area and adjacent areas) and remotely (e.g. in the intestine and the lung) (6–9). Second, the peroxynitrite/PARP axis plays a pathogenetic role in an ovine model of burn injury, because pharmacological neutralization of peroxynitrite or inhibition of the catalytic activity of PARP improves the outcome (10,11). Third, pharmacological inhibition of PARP has been shown to improve pulmonary and intestinal function in rodent models of burn injury (8,12).

The goal of the present study was to extend the investigation of PARP activation in burns to human subjects. Skeletal muscle biopsies, obtained at various stages of burn injury, are immensely useful to study changes in gene expression, metabolism and inflammatory responses (13,14). In the present study, we have investigated potential changes in PARP activity in human burns, and studied the time-course of this response. An additional aim was

to conduct tissue localization studies, in order to identify the cell types that show the most pronounced activation of PARP. Since several studies demonstrated that treatment of burn patients with propranolol provides beneficial effects (15), we also have tested the potential effect of propranolol therapy on PARP activation.

Methods

Collection of muscle biopsy samples from children with severe burn injury

Children aged 0 to 17 years with more than 40% total body surface area burns that would require skin grafting, who arrived at our hospital within 96 hours of injury, were eligible. All subjects received standard burn care as previously described (16). Each patient underwent wound excision and grafting with skin autografts and allografts within 72 hours of admission. Sequential grafting procedures were performed over time until the wounds were 95% healed. Enteral nutrition was started at admission and continued until the wounds were 95% healed. Patients were fed a commercial enteral formula (Vivonex T.E.N.; Sandoz Nutritional, Minneapolis, MN) through a nasoduodenal tube. The daily caloric intake was calculated to deliver 1500 kcal per square meter of body-surface area burned plus 1500 kcal per square meter of total body surface area. Patients remained in bed for 5 days after each excision and grafting procedure and then were allowed daily walks. All patients were administered antianxiety medication after the first week postburn. Biopsy of the vastus lateralis muscle was taken at various times postburn and pooled into three groups: Early (samples taken at 2–6 days post-burn; n=4), Middle (13–18 days post-burn; n=4) and Late (69–369 days; n=8). Biopsies from cleft-lip and cleft palate patients between 3 and 18 years of age admitted to our hospital for reconstructive surgery were used as nonburned normal controls (n=3). Collection and analysis of the samples occurred with the approval of the institutional IRB committee. Patients were randomized to receive propranolol or no propranolol treatment, as part of a prospective randomized clinical trial; samples (homogenates of skeletal muscle biopsies suitable for Western blotting analysis, or frozen samples suitable for immunohistochemical analysis) that were collected over a period of 2 years were obtained from a tissue bank for the current study. All samples that contained suitable volume were analyzed; no samples were excluded from the analysis. Patient demographic data are shown in Table 1. Biopsies were snap frozen at -80°C for subsequent Western blot analysis or for immunohistochemical analysis.

Propranolol treatment

The drug was given in a regimen as previously described (17), at 4 mg/kg/day by mouth from the time of admission for a period of 10 ± 1 months. Patients were closely monitored for heart rate and blood pressure. Patients did not receive any other anabolic or anticatabolic agent. During the in-hospital portion of the treatment, patients received insulin if necessary (blood glucose >210 mg/dl) to decrease blood glucose below 210 mg/dl, with target blood glucose of 140 to 160 mg/dl. Pharmacokinetic studies demonstrated that in the current patient population the effective plasma drug concentrations were achieved in 30 minutes, and the half-life is approximately 4 hours (18). Skeletal muscle biopsies from the propranolol-treated patients, obtained in the 'Middle' and 'Late' time points (6–19 days post-burn; n=4 and 139–289 days post-burn, n=5, respectively) were compared with the responses seen in the respective comparable groups of control patients (not treated with propranolol).

Western blotting for poly(ADP-ribose) (PAR)

Muscle samples were homogenized in homogenizing buffer (50mM Tris pH 7.4, 150mM NaCl, 1% Triton-X-100, 10mM EDTA, Protease Inhibitor Cocktail (CompleteMini by Roche). PAR Western blotting was performed as previously described (19). Proteins were

loaded onto 4–12% polyacrylamide gels and separated by electrophoresis then transferred to nitrocellulose membranes. For immunoblotting, membranes were blocked with 10% nonfat dried milk in Tris-buffered saline (TBS) for 90 min. The primary antibody (anti-PAR (poly-ADP-ribose) polyclonal antibody, EMD Biosciences) against PAR were applied at 1 µg/ml concentrations overnight at 4°C. After washing 3 times in TBS containing 0.05% Tween-20 (TBST), the secondary antibody (peroxidase-conjugated goat anti-rabbit) were applied at 1:2000 dilution for 1 h. Blots were washed 3 times in TBST, once in TBS, and incubated in enhanced chemiluminescence reagents (Supersignal WestPico Chemiluminescent substrate; Pierce Biotechnology, Rockford, IL, USA). The levels of PARylated protein at 120 kDa (representing auto-PARylation of PARP-1) were normalized to actin.

Immunohistochemical analysis

Frozen sections of 5 micron thickness were prepared from all biopsy specimens collected. Sections were fixed in cold 95% ethanol for 10 minutes, then immersed in 3% hydrogen peroxide solution for 10 minutes, and rinsed with de-ionized water. Slides were processed at room temperature in a Dako horizontal auto-stainer, using the biotin-streptavidin method. Both avidin and biotin were obtained from Vector Laboratories, as part of the AB blocking kit, and diluted 1:5 using Dako antibody diluent. Tris buffered saline was used to rinse slides between each of the consecutive processing steps. The primary antibodies were diluted in the biotin solution, each to the concentration specified as follows, and applied for 1 hour. Primary antibodies used in our studies were: 1) PAR (Tulip mouse monoclonal, anti-human 1:10); 2) CD-31 (DAKO mouse monoclonal, anti-human, 1:200); 3) Von Willebrand factor (DAKO mouse monoclonal, anti-human, 1:200); S-100 (DAKO rabbit polyclonal, anti-human, 1:4000).

PAR immunostaining procedure—Sections were incubated with diluted avidin for 7 minutes, rinsed, and incubated with the primary antibody (PAR) biotin solution for 1 hour. Afterwards, slides were incubated in universal secondary antibody LSAB2 (Dako) for 15 minutes, followed by LSAB2 labeling agent (Dako) for 15 minutes, and then diaminobenzidine (DAB, Dako) for 5 minutes. Slides were rinsed in distilled water, counterstained with Harris Hematoxylin (Fisher Scientific) for 1 minute, rinsed in distilled water first, 0.25% ammonia water, and distilled water as final step. Slides were then dehydrated through graded series of alcohols, four changes of xylene, and finally coverslipped with synthetic glass and permount mounting media.

Double immunostaining procedure—Double immunohistochemical staining was performed according to the following combinations: 1) PAR (FR) and CD-31 (DAB), 2) PAR (FR) and factor VIII-related antigen (DAB), 3) PAR (FR) and S-100 (DAB). Sections were incubated with diluted avidin for 7 minutes, rinsed, and incubated with the primary antibody (CD-31, fVIII-related antigen, or S-100) biotin solution for 1 hour. Afterwards, slides were incubated with PAR antibody solution (10 microg/mL) for 1 hour, and then in universal secondary antibody LSAB2 (Dako) for 15 minutes, followed by LSAB2 labeling agent (Dako) for 15 minutes, and then diaminobenzidine (DAB, Dako) for 5 minutes. At this point, slides were incubated with the tertiary antibody solution alkaline phosphatase streptavidin (1:200) for 15 minutes, and then the fast-red chromagen (Biopath Labs) was applied for 5 minutes. Slides were finally rinsed, counterstained, dehydrated and coverslipped as described above.

Statistical analysis

Nonparametric ANOVA test was applied for statistical analysis and for the determination of significance, the Kruskal-Wallis post-hoc test was used. $P < 0.05$ considered as significant.

Results

Normal skeletal muscle biopsies showed very low levels of PARP activation, as evidenced by PAR Western blotting (Figs. 1–2). The samples obtained at the 'Early' post-burn time point tended to increase in PARylation, representing an approximate doubling of the PAR signal (Fig. 2). The samples obtained at the 'Middle' time point showed the most pronounced degree of activation, representing an approximately 5-fold increase in PAR signal ($p < 0.05$) (Fig. 1). The samples obtained at the 'Late' time point showed a heterogeneous response, and tended to remain elevated compared to healthy controls (Fig. 2). The major band showing PARP activation in burns was the 120 kDa band, which is consistent with auto-PARylation of PARP-1, the major PARP enzyme. However, several additional, PARylated higher molecular-weight bands were also detected after burn injury (Fig. 1).

Immunohistochemical evaluation of frozen sections collected from the patients of the Middle group showed the most pronounced PARP activation. In the control section examined, no positive staining was observed (Fig. 3A). PAR immunostaining was primarily observed within cells of the capillary endothelium (Fig. 2B, C, D). This observation was confirmed by performing double immunostaining to show the co-localization of PAR with both CD-31 (Fig. 2E) and factor VIII-related antigen (Fig. 2F). As shown in detail in Figure 2C, E, and F, no PAR-positive staining was observed within the nuclei of myocytes. In a few sections, positive PAR staining was observed in scattered mononuclear cells, and also in very rare neuroglial cells, which were confirmed to be Schwann cells by concomitant S-100 immunostaining.

We have also tested the effect of propranolol therapy on the degree of PARP activation. Propranolol treatment markedly and significantly ($p < 0.05$) reduced the degree of PARP activation in the Middle group of patients (Figs 1–2); also the samples obtained in the 'Late' group of patients tended to be lower in the propranolol group than in the group of patients who did not receive propranolol (Fig. 2).

Discussion

Prior to the current investigation, there were no published studies investigating potential changes in PARP activity in *human* burn conditions. Nevertheless, there were several lines of data suggesting that oxidative and nitrosative stress (upstream processes in PARP activation in various forms of critical illness) is a key feature of human burns: a study in children with burns showed an increase in plasma lipid peroxidation and a decrease in total plasma antioxidant capacity and antioxidant vitamin levels (20–22); and increased reactive oxidant species formation and increased plasma xanthine oxidase levels were reported in patients with burns, especially in the non-survivor group (23). In the context of the above findings, we hypothesized that activation of PARP (as a result of the formation various reactive oxygen and nitrogen species) may occur in human burns. The findings presented in the current report demonstrate that this is, indeed, the case: burn injury induces a marked activation of PARP in the parenchymal tissue studied (skeletal muscle biopsy). Although the majority of the cells in this biopsy consist of myocytes, the activation of PARP was primarily localized to endothelial cells, and occasionally to resident mononuclear cells. It is conceivable that during burns PARP activation may not only occur in the endothelial cells of the skeletal muscle vasculature, but also in the vasculature of other organs, representing a systemic response. However, this remains to be directly investigated in subsequent studies.

The time-course of the response was somewhat unexpected, as we have hypothesized that the peak of the response would occur in the early stage of the disease (a few days after the

burn injury), because it is this stage of the disease which is typically associated with the highest degree of reactive oxidant species production (20–23), which is typically viewed as an immediate/early response to the acute stage of the burn injury. The data, however, indicated, that the peak of the response was in the 'Middle' stage of the disease (several weeks after burns), although in some patients PARP activation tended to remain high even at prolonged periods of time (up to a year after the burn injury). Although we cannot identify the molecular triggers of PARP activation in our patients, it is noteworthy in this context that recent studies have demonstrated that the depletion of the antioxidant vitamins alpha and gamma-tocopherol in burned pediatric patients occurs in a period of several weeks to months (24), which does coincide with the period where we have observed marked PARP activation in the current study. It is conceivable that some degree of oxidant formation and/or antioxidant depletion continues in the middle stage of the disease in burn (or possibly even until the late stage of the disease), which, in turn, makes the endothelial cells more susceptible to oxidative injury and PARP activation.

What role, then, does PARP activation play in the pathobiology of human burns? Based on the results of preclinical studies, where pharmacological inhibitors of PARP were tested in rodent or large animal models of burns (11,12), we hypothesize that PARP activation is part of a deleterious, suicidal tissue response, which may contribute to the pathogenesis of burn-associated alterations such as (a) local tissue injury (b) remote tissue injury and (c) dysregulation of the inflammatory and immune responses. Based on the role of PARP activation in endothelial cells (as studied in cell-based models, preclinical studies and correlation analysis of human vascular responses and PARP activity) (26–29) we hypothesize that PARP activation in endothelial cells during burns may lead to endothelial cell dysfunction, dysregulation of local vascular control, or may trigger the infiltration of mononuclear cells into various organs. Several lines of studies have also implicated PARP in the regulation of angiogenesis; PARP inhibitors suppress the angiogenic response (30–32). Consequently, one may also hypothesize that PARP activation in endothelial cells during burns may contribute to endothelial cell proliferation, migration and tube formation, and may be part of the angiogenic response that promotes wound healing. These mechanisms remain to be further elucidated in follow-up investigations.

An important goal of our studies was to test the effect of propranolol therapy on PARP activation on burns. The rationale of these studies was as follows. Several studies demonstrated that treatment of burn patients with propranolol provides beneficial effects, and most of these effects were attributed to the control of the hypermetabolic response (15, 18, 33). There may be several mechanisms by which propranolol may modulate oxidative/nitrosative stress pathways and PARP activation. (1) Because propranolol affects the overall metabolic status of the patient (as reviewed in 34) it is conceivable that it may affect the body's free radical and oxidant production (which, at least in part, is the byproduct of mitochondrial processes), consequently, reducing the oxidant trigger of PARP activation. (2) In addition, propranolol exerts anti-inflammatory and direct antioxidant effects (35,36) and attenuates pro-inflammatory mediator production in burns (37). Given the role of PARP as an enhancer of pro-inflammatory responses in various cell types, we speculate that inhibition of PARP activation by propranolol may contribute to a 'switch' to an anti-inflammatory phenotype in propranolol-treated burn patients.

Propranolol treatment of burn patients improves metabolic function in burns and reduces muscle wasting (15–18, 34). We have not found any evidence for PARP activation in skeletal muscle myocytes, and there are no publications on physiological or pathophysiological roles of PARP in the regulation of skeletal muscle metabolism. Nevertheless, one can speculate that the prevention of PARP activation in leukocytes of propranolol-treated patients may produce an anti-inflammatory phenotype (see above),

which may also affect muscle wasting. Furthermore, PARP activation in endothelial cells is known to contribute to endothelial dysfunction (2–4, 25–30), and inhibition of PARP activation by propranolol may improve the hemodynamic status of the skeletal muscle, which may be beneficial for the skeletal muscle metabolic status. These possibilities need to be directly tested in future studies.

Changes in gene expression after burns have previously been investigated in a comparable cohort of pediatric burn patients using Affimetrix gene chip analysis (14). Because PARP (a constitutively expressed enzyme) in critical illness is regulated on the catalytic level of the enzyme and not on the level of its mRNA expression, it is not surprising that changes in this enzyme were not detected in this analysis. However, it is noteworthy that this analysis has demonstrated significant changes in the expression of a number of enzymes at all time points of the disease (Early, Middle and Late), with diverse functions including signaling, proliferation, metabolism and others. No changes were, however, reported in oxidant/antioxidant enzymes or NO synthases, enzymes that are known to lay upstream from PARP activation in various human diseases (2). Therefore, further studies (genetic and biochemical) will be needed to identify the exact molecular triggers of PARP activation at various stages after burns.

In summary, the current study provides evidence for the activation of PARP in human patients with severe burn injury; delineates the time-course of the response; and demonstrates the protective effect of propranolol. In order to clarify the exact mechanistic role of PARP in human burns, future, interventional studies with PARP inhibitors would be necessary. In recent years, several potent inhibitors of PARP have entered the stage of clinical testing (e.g. 38–41); other trials have utilized nicotinamide, the endogenous 'feedback' inhibitor of the PARP enzyme (e.g. 42). Furthermore, recent studies suggest that certain PARP polymorphisms may affect the outcome of various forms of critical illness (43,44). In summary, the field of PARP has entered the clinical stage, which underlines the timeliness of additional translational studies in various areas including burns and various forms of critical illness.

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Abbreviations used

eNOS	endothelial nitric oxide synthase
iNOS	inducible nitric oxide synthase
NAD⁺	nicotinamide adenine dinucleotide
NO	nitric oxide
O₂^{•−}	superoxide
ONOO[−]	peroxynitrite anion
PAR	poly(ADP-ribose) polymer
PARP	poly (ADP-ribose) polymerase

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Fig. 1.

Representative PAR Western blots are shown from homogenates of skeletal muscle biopsies of control patients (no burn, n=3), of patients with burn injury at the middle stage of the disease (n=4) and of propranolol-treated patients with burn injury at the middle stage of the disease (n=4). The bottom part of the figure shows the corresponding actin loading control bands.

Fig. 2.

Time course of PARP activation in skeletal muscle biopsies of pediatric burn patients and reduction of PARP activation in skeletal muscle biopsies of pediatric burn patients treated with propranolol. Densitometric analysis of PAR staining, normalized to actin, is shown, in control subjects (no burn injury), and in the Early, Middle and Late stage of the disease in the absence of propranolol treatment, and the Middle and Late stage of the disease in the presence of propranolol treatment. Results are expressed as means±SEM. * $P<0.05$ shows a significant increase in the PAR response, when compared to the control group in the 'Middle' group of patients; # $p<0.05$ shows a significant inhibition of the response by propranolol in the 'Middle' group of patients.

Fig. 3.

High-power photomicrographs of frozen tissue sections studied by immunohistochemistry to show the activation and localization of PAR in 'Middle' group of patients. **A:** Control tissue sections, showing no significant PAR staining. Original magnification: 40×. **B:** Tissue sections collected from a burn case, showing marked activation of PAR. Original magnification: 20×. **C–D:** Tissue section collected from a burn case, showed at higher magnification, highlighting the localization of PAR-positive staining within cells of the capillary endothelium (brown staining), while the nuclei of myocytes appear negative (blue counterstaining). Original magnification: (C) 40×, (D) 100×. **E–F:** Tissue sections collected from a burn case showing red staining for PAR and brown staining for either CD-31 (E) or Factor VIII-related antigen (F), to demonstrate co-localization within vascular endothelial cells. Original magnification: (E, F) 100×.

TABLE 1

Demographic Characteristics of the Study Groups

	Control	Early	Middle	Late	Middle +Propranolol	Late +Propranolol
Subject number	3	4	4	8	4	5
male	2	2	1	7	4	4
female	1	2	3	1	0	1
Age	11.6±1.2	8.8±3.2	9.5±3.3	5.5±1.6	11.2±1.8	9.4±2.3
Burn (TBSA), %	n/a	62±5	75±6	66±5	70±8	68±7
Height (kg)	48±3	47±20	56±20	33±8	52±12	44±12
Weight (cm)	159±12	135±15	132±17	123±11	148±13	134±16
Samples obtained post-burn (days)	n/a	3±1	15±2	203±40	13±3	233±30

Values are mean ± SEM except where otherwise noted. Height and weight measured at baseline. All 4 groups of burned children were similar in demographics. n/a= non applicable.