Turkish Neurosurgery 2008, Vol: 18, No: 2, 149-156

Effects of Peripheral Nerve Ischemia-Reperfusion Model on Serum Cytokine Levels*

Periferik Sinir İskemi Reperfüzyon Modelinin Serum Sitokin Düzeylerine Etkisi

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ABSTRACT

AIM: Although the neuropathology of ischemic nerve fiber degeneration is relatively well known, its pathogenesis is poorly understood. Local cytokines, which have neuroprotective effects on inflammation and repair, participate in the process by undefined mechanisms. In this study, we evaluated the effects of ischemia and reperfusion on the sciatic nerve of the rat and investigated the probable effects of cytokines on this period.

MATERIAL and METHODS: In the current study, ischemia and reperfusion injury of sciatic nerve was rendered by clamping the femoral artery and vein of the rat for three hours and was followed by varying durations of reperfusion. Activin A, TGF ß1 and TGF, ß2 levels were measured in serum samples.

RESULTS: TGF ß1 and Activin A were found to be increased in the ischemic groups compared with the control group ($p<0.05$). A significant difference was found between the experimental groups after reperfusion ($p<0.05$). There was no statistical significance for TGF ß2 levels between the study groups ($p>0.05$).

CONCLUSION: Ischemia causes some important changes in biochemical parameters, and nerve injury continues for a while according to the reperfusion time. Ischemia-reperfusion injury of peripheral nerves caused by various reasons therefore affects the levels of cytokines.

KEY WORDS: Activin A, Ischemia–reperfusion injury, Peripheral nerve, Transforming growth factor beta-1, Transforming growth factor beta-2.

ÖZ

AMAÇ: İskemik sinir hasarının nöropatolojisi, göreceli olarak, iyi bilinmesine karşın patogenezi yeterince aydınlatılamamıştır. İnflamasyon ve tamir olaylarında nöroprotektif etki gösteren lokal büyüme faktörleri yani sitokinler de tam olarak açıklanamayan mekanizmalarla bu sürece katılmaktadırlar. Bu çalışmada, iskemi-reperfüzyon hasarının rat siyatik siniri üzerindeki etkileri değerlendirildi ve sitokinlerin bu dönemdeki olası etkileri incelendi.

YÖNTEM ve GEREÇ: Bu çalışmada, iskemi reperfüzyon hasarı, rat femoral arter ve veninin 3 saat süreyle klempe edilmesi ve ardından değişen sürelerde reperfüzyona bırakılması ile sağlandı. Serum örneklerinde Aktivin A, TGF b1 ve TGF b2 düzeyleri ölçüldü.

BULGULAR: İskemik gruplarda TGF b1 ve Aktivin A serum düzeylerinin kontrollere göre artmış olduğu saptandı ($p<0,05$). Reperfüzyon sonrasında ise çalışma grupları arasında anlamlı fark olduğu gözlendi ($p<0,05$). TGF b2 düzeyleri açısından çalışma grupları arasında istatistiksel olarak anlamlı fark izlenmedi ($p>0,05$).

SONUÇ: İskemi nedeniyle biyokimyasal parametreler değişmektedir ve reperfüzyon süresine bağlı olarak sinir hasarı da bir süre devam etmektedir. Bu nedenle, farklı sebeplerle gelişen periferik sinir iskemi-reperfüzyon hasarının sitokin düzeylerini etkilediği düşünülebilir.

ANAHTAR SÖZCÜKLER: Aktivin A, İskemi-reperfüzyon hasarı, Periferik sinir,Transforme edici büyüme faktörü beta-1,Transforme edici büyüme faktörü beta-2.

*A report of this work has been presented in abstract form at the 30th FEBS Congress & 9th IUBMB Conference, 2-7 July 2005, Budapest, Hungary.

Received: 22.02.2008
Accepted: 01.05.2008

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INTRODUCTION

A peripheral nerve has two basic requirements to fulfill its functions normally: The nerve must be connected to the cellular body in the central nervous system, and it must have a continuous and sufficient oxygen supply through the intraneural system. While the degree of the neural tissue damage that occurs during ischemia is related to the duration of the ischemia and the blood flow during this period (8), the cellular and biochemical interactions which emerge as a result of the provision of flow back to the damaged tissue may aggravate the tissue damage. This paradoxical aspect of resuming blood flow is known as reperfusion damage (43).

Although several studies have been published in the last few years on the neuropathology of peripheral nerve ischemic degeneration, the pathophysiological mechanisms are not fully elucidated. It is argued that ischemic cell damage leads to inflammation and inflammatory mechanisms play a major role in the pathogenesis of the ischemia/reperfusion (I/R) damage. Leukocytes, leukocyte adhesion molecules and cytokines take part in these inflammatory mechanisms (5). Infiltrated macrophages play a crucial role in the process of Wallerian degeneration by removing axonal and myelin debris (3) and producing cytokines that stimulate production of neurotrophic factors by non-neuronal cells of the distal nerve stumps (9). Transforming growth factor, (TGF ß) is among the most widespread and versatile cytokines in the nervous system (4,41). TGF ß is secreted into the injured nerves by invading macrophages (2) and by Schwann cells themselves after nerve injury (34).

In addition to their neuroprotective effects, cytokines are also potent inducers of many extracellular matrix components such as fibronectin, collagen and cell surface integrins (22,26,35,39).

Activins are dimeric proteins belonging to the TGF ß superfamily. There are reports on the roles of activins in growth and differentiation, organogenesis, inflammatory events and injury recovery (26). High activin A expression has been shown to accompany the acute neuronal damage of various origins in many acute brain damage models (26). Activin A modulates the survival period of damaged neurons and has a neuroprotective effect against traumatic brain damage (1,22,26,27,28).

There are few studies on the response of peripheral nerve to ischemic damage and reperfusion. The aim of the current study was to determine the possible roles of local growth factors, TGF ß1, TGF ß2, and activin A in peripheral nerve damage, and to describe their relations.

MATERIALS and METHODS

This study was undertaken with the approval of the Ethics Committee of the Faculty of Medicine, University of Mersin.

Male Wistar albino rats (250–275 g), obtained from the Animal Laboratory of the Faculty of Medicine, Selcuk University (Konya, Turkey), were used in this series of experiments. The animals were acclimatized for 1 week to the conditions of our laboratory before commencement of the experiment. The animals were exposed to a 12-hour light and 12-hour dark cycle at a room temperature of 22 oC. The animals had free access to standard laboratory chow and water ad libitum. The rats were divided into nine groups: Ischemia and reperfusion were not applied to the control group. Only ischemia was performed but reperfusion was not established in the ischemia group. For the other groups 1, 2, and 24-hours, and 1, 2, 3 and 4-weeks of reperfusion was applied following 3-hours of ischemia. Study groups are given in Table I. Eight rats were randomly allocated to each group. The rats were anesthetised by intraperitoneal injection of ketamine HCl (50 mg/kg) (Ketalar®Eczacıbası, Warner Lambert Ilac AS, Istanbul, Turkey) and xylazine (5 mg/kg) (Rhompun® Bayer AG, Leverkusen, Germany). The animals were placed in the supine position on a heated mat during the operation and recovery. Right femoral vessels were exposed through an inguinal incision and were dissected free from the femoral nerve with a surgical loop. The trifurcation of the sciatic nerve into peroneal, tibial and sural branches was rendered almost completely ischemic by occluding the femoral artery and vein with a Yasargil microvascular clamp (Standard aneurysm clamp, FE751, Aesculap, Tuttlingen, Germany) for 3 hours as described by Saray et al (36). After the end of the reperfusion, blood samples of 5 ml were obtained from the heart under anesthesia. Blood sampling subsequently led to the death of the animal.

Biochemical preparation and evaluation

An acid treatment procedure was applied to the serum samples for TGF ß1 and TGF ß2 analysis.

Table I: General Details of Experimental Protocol

Groups	Ischemia	Duration of ischemia	Reperfusion	Duration of reperfusion
Control	-	-	-	-
Ischemia	+	3 hours	-	-
1h	+	3 hours	+	1 hour
2h	+	3 hours	+	2 hours
24 h	+	3 hours	+	24 hours
1w	+	3 hours	+	1 week
2w	+	3 hours	+	2 weeks
3w	+	3 hours	+	3 weeks
4w	+	3 hours	+	4 weeks

Since the kits used in the study, TGF ß1 (E max immunoassay system 145822 Promega corporation, Madison, WI, USA) and TGF ß2 (E max immunoassay system 143540 Promega corporation, Madison, WI, USA), were designed to measure the biologically active quantities that are immunoreactive, this procedure was applied in order to measure the total quantities. In this procedure, the samples were diluted in a 1/5 ratio using DPBS (Dulbecco's phosphate-buffered saline) buffer. Next, 2 μl of 1 N HCl were added to each 100 μl sample and the pH was checked to ensure that it was equal to or below 3. The samples were mixed well and incubated at room temperature for 15 minutes. This was followed by adding 2 μl of 1 N NaOH to each 100 μl sample for neutralization and the pH was checked to ensure that it was approximately 7,6.

TGF ß1 assay: The TGF ß1 E max immunoassay system 145822 (Promega corporation, Madison, WI, USA) was used. The same method was employed for all samples and standards. Following the change in color, the absorbance values were read by using an ELISA reader at a wavelength of 450 nm. The absorbance values were converted to concentration by reference to a standard curve. The results were expressed as ng/ml of serum.

TGF ß2 assay: The TGF ß2 E max immunoassay system 143540 (Promega corporation, Madison, WI, USA) was used. The same method was employed for all samples and standards. The color change from blue to yellow was observed and a reading performed using an ELISA reader at a wavelength of 450 nm. The absorbance values were converted to concentration by reference to a standard curve and expressed as ng/ml of serum.

Activin A assay: In this study, activin A measurement was performed using an Activin A immunoassay kit (Quantikine M Murine, Mouse/Rat Activin A Immunoassay, 0141148 (R&D systems, Minneapolis, MN, USA). The same method was employed for all samples and standards. The absorbance values were read using the ELISA reader at a wavelength of 450 nm, and simultaneously at the reference wavelength of 540 nm for wavelength verification. The absorbance values obtained were converted to concentration by using a standard curve. Values were expressed as pg/ml of serum.

STATISTICAL ANALYSIS

The statistical package SPSS for Windows (version 9.05) was used for statistical analysis. Descriptive analysis was performed for calculations of the mean and standard error of the mean (SEM) for all variables, and for each study group. One-way variance analysis (one-way ANOVA and Tukey post hoc tests) was performed for differences between the experimental groups for the same variable. A correlation analysis was performed for assessment of relationships between within-group variables. Significance (p) was assumed at $p<0,05$. A significant correlation (r) was assumed when $p>0,50$.

RESULTS

Means and standard errors of TGF ß1, TGF ß2, and activin A levels for each study group are given in Table II and multiple comparisons are shown in Figures 1-3.

TGF ß1 Results

Statistical examination of TGF ß1 study revealed a difference between study groups with respect to TGF ß1 values, with a significance of $p=0.000$.

Table II: Activin A, TGF ß1 and TGF ß2 Levels of All Study Groups*

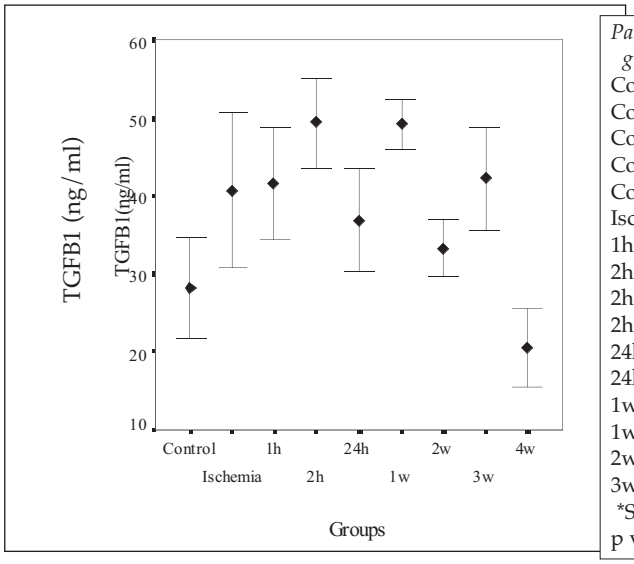
Groups	n	Activin A (pg/ml) Mean±SEM	TGF ß1 (ng/ml) Mean±SEM	TGF ß2 (ng/ml) Mean±SEM
Control	7	514.29 ±33.90	28.16 ± 2.60	3.55 ± 0.46
Ischemia	8	595.16 ±28.99	40.68 ± 4.19	4.31 ± 0.54
1h	8	835.50 ±89.59	41.59 ± 3.07	4.33 ± 0.21
2h	8	626.05 ±39.28	49.34 ± 2.49	5.18 ± 0.85
24h	8	743.93 ±34.38	36.87 ± 2.82	4.34 ± 0.29
1w	8	606.56 ±54.91	49.16 ± 1.33	6.12 ± 0.56
2w	8	876.15 ±90.39	33.26 ± 1.59	5.14 ± 0.89
3w	8	604.69 ±62.41	42.17 ± 2.75	4.09 ± 0.40
4w	8	738.66 ±36.23	20.57 ± 2.15	3.98 ± 0.43

**SEM, Standart error of mean; TGF ß1, Transforming growth factor beta 1; TGF ß2, Transforming growth factor beta 2; h, hour; w, week.*

The study groups were compared to each other in terms of TGF ß1 results, and a difference was found between the groups. Statistically significant pairwise groups are shown in Figure 1.

TGF ß2 Results:

Statistical significance was not found between the study groups for TGF ß2 levels (p=0.074). The pairwise groups were not statistically significant in TGF ß2 analysis (Figure 2).



Pair wise groups	P values
Cont-Isch	0.050*
Cont-1h	0,027*
Cont-2h	0,000*
Cont-1w	0,000*
Cont-3w	0,017*
Isch-4w	0,000*
1h-4w	0,000*
2h-24h	0,038*
2h-2w	0,002*
2h-4w	0,000*
24h-1w	0,043*
24h-4w	0,002*
1w-2w	0,002*
1w-4w	0,000*
2w-4w	0,033*
3w-4w	0,000*

**Statistically significant p values*

Figure 1: *TGF ß1 results and multiple comparisons of study groups. (Dots represent mean values given in Table 2, and bars represent 95% CI).*

Activin A Results:

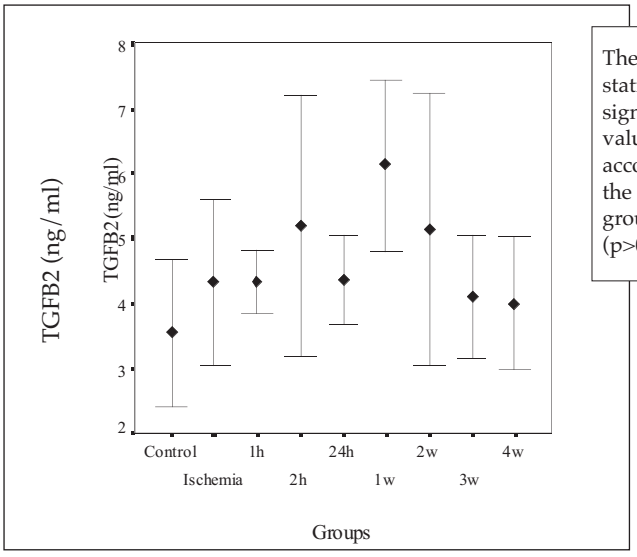
A significant difference (p=0.000) was found between the study groups with respect to activin A values.

When the study groups were compared to each other in terms of activin A results, a difference was found between some of the groups. Statistically significant pairwise groups are shown in Figure 3.

A correlation analysis was used in the evaluation of relationships between TGF ß1, TGF ß2, and activin A within the same group. A linear relationship between TGF ß1 and TGF ß2 was observed (p=0,041, r=-0,727) in the 3-weeks group, while no direct relationship was observed between the biochemical parameters in the other groups.

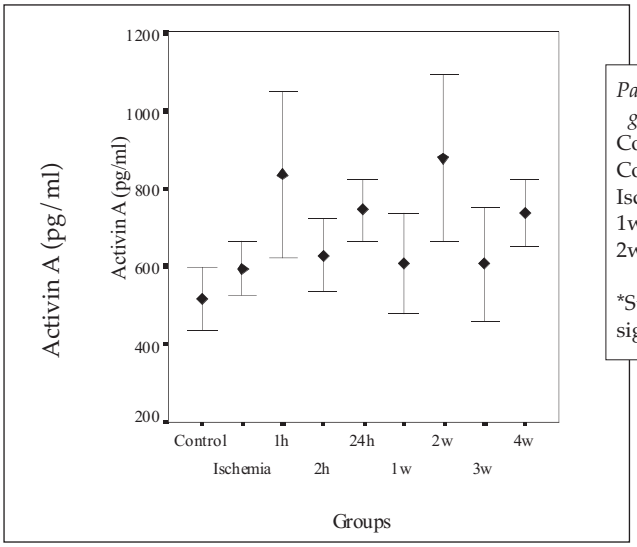
DISCUSSION

Although mechanisms for nerve injury after ischemia and reperfusion are still unclear, a number of candidate mechanisms have been identified. Peripheral nerves are more resistant to ischemia than brain tissue since they have lower energy requirements, contain an extraneural and intraneural



There was no statistically significant p values according to the pair wise groups (p>0.05).

Figure 2: *TGF ß2 results. (Dots represent mean values given in Table 2, and bars represent 95% CI).*



Pair wise groups	*P values*
Cont-1h	0,008*
Cont-2w	0.002*
Isch-2w	0,024*
1w-2w	0,036*
2w-3w	0,033*

*Statistically significant p values

Figure 3: *Activin A results and multiple comparisons of study groups. (Dots represent mean values given in Table 2, and bars represent 95% CI).*

common vascular anastomosis, and have the ability to employ anaerobic metabolism (15,20,21). Injury to neurons results in some molecular and cellular responses that are associated with a successful regenerative response to maintain recovery. As Makwana M. and Raivich G. discuss in their review, transcription factors, adhesion molecules, growth-associated proteins and structural components needed for axonal elongation are induced as a response to injury in the injured neuron (23).

It is argued that the post-ischemia reperfusion phenomenon and decreased blood flow are caused by persistent leukocyte adhesion (25,37). Leukocytes interact with cytokines, increasing the synthesis of adhesion molecules in various cells such as endothelial cells. Thus, the inflammatory response is initiated in the damaged tissue (30). The expression of adhesion molecules and infiltration of leukocytes to the nerve must increase for a neural inflammatory response (7). Leukocytes therefore play a crucial role in the neural inflammatory response. Axonal injury causes acute inflammatory changes including the influx of leukocytes at the lesion site in peripheral nerves. When local inflammatory molecules are activated, they move into direct contact with the cell body to interact with T cells that are already present in the injured neural tissue (16).

Although increases and decreases in cytokine levels were observed depending on the reperfusion period in the all I/R groups in the present study, these serum results were found to be higher than the control values. According to these variable levels of cytokines in different study groups, these molecules may play a role in peripheral nerve regeneration.

The members of the TGF family are potent regulators of cell growth and differentiation, inflammatory events, extracellular matrix formation and wound healing (14). Although TGF ß1 immunoreactivity is weak, or even absent in the normal sciatic nerve, TGF- ß1 protein and mRNA levels increase after a nerve lesion (12,33,38,42). TGF ß has been shown to play a protective role against I/R damage in different organ systems but we have very little knowledge on the regulation of TGF ß activity in this event (24). In a study conducted on a different tissue, levels of all growth factors, especially those of TGF ß, were increased following I/R (6). This increase was found to be rather high compared to the control value after 6-hours reperfusion (6). An increase in TGF, regulation in the nerves was shown in another study (11). TGF ß is an important regulator in the life cycle of neurons and also has a neuroprotective effect (1,17). TGF ß therefore emerges as a protective molecule against I/R damage in later stages of reperfusion (24).

In the current study, we observed that TGF ß1 levels were higher than the controls both during ischemia and all reperfusion periods. Although increases and decreases in TGF ß2 were similar to TGF ß1, a statistical significance was not found between the study groups for TGF ß2 levels. Both these parameters reached their peak in the 2-hours and 1-week reperfusion periods. There was a correlation between TGF ß1 and TGF ß2 (r =-0,727) in the 3-weeks reperfusion period, and these molecules may have a role in repair at late stages of reperfusion. The results of another report, which are consistent with our findings, suggest that antagonism of TGF ß1 functions in the acute phase following spinal cord injury may exacerbate the degree of injury (13). As discussed in several studies, anti-TGF ß1 and anti-TGF ß2 have similar effects on functions such as Schwann cell mitosis and glial scar formation (13,18,19,32). However, TGF ß1 and TGF ß2 show different functions in other areas such as inflammation following injury. It has been shown that TGF ß1 acts in several ways to suppress activities of microglia and their associated inflammatory functions in a way that has not been reported for TGF ß2 (31). Nakamura et al. showed that the rate of induction of TGF ß1 following spinal cord injury correlates with the rate of appearance of anti-inflammatory cytokines (29). The authors suggest that the detrimental effects of anti-TGF ß1 were due to blockade of an anti-inflammatory effect of TGF ß1 not shared by TGF ß2 (13).

TGF ,1 levels reached their peak after 2 hours and 1 week, and than returned to control levels at 4 weeks in this study. Another study, where the sciatic nerve was sutured after transection to prevent axonal regeneration, reported that TGF-ß1 mRNA expression in the endoneurium of the distal stump reached its peak after 2 weeks, and at weeks 3-6, and that the expression was two to four times higher than in the controls. These results are not consistent with our results as the nerve injury procedure was different. Furthermore in our study TGF ß1 measurement was performed in serum samples, although Taskinen HS et al studied the expression of TGF ß1 mRNA (40). They also reported that the expression of TGF-ß1 mRNA in the epineurium of proximal stump was over 700 times higher than that found in the non-operated controls (40). For our last study group, i.e. 4-weeks reperfusion period, no relation was observed between the parameters (r<0,50), and this may imply that the nerve has started to recover.

Another member of TGF ß superfamily, Activin A, is a potent stimulator that plays a role in injury recovery. It plays an important role in the early cellular response in neuronal damage. Activin mRNA and protein expression increase significantly several hours following the damage (28). There are reports that activin A administered exogenously may protect neurons against acute cell death (28).

Activin has been reported to decrease in in vitro ischemic brain damage, prolong the life cycle of neurons, and protect neurons against toxic damage in infant rats (44). An increase in activin A regulation was observed in an acute brain damage model (10). We found that activin A levels were higher compared to the controls during both ischemia and all reperfusion periods in our experimental model where we induced I/R damage in the peripheral nerve. These values reached their peaks in the 1-hour, 24-hours, and 2-weeks intervals. According to the activin A serum levels affected by the I/R of the peripheral nerves, this molecule may be thought to be play a role in the probable mechanisms of tissue repair. To the best of our knowledge, the present study investigating the probable effects of activin A on I/R injury is the first in the current English literature.

In conclusion, the findings obtained within the framework of this model indicate that the levels of cytokines are affected in case of I/R which may arise due to various reasons, and that these molecules may take part in the formation and/or repair of the damage. I/R injury is accompanied by the synthesis of proinflammatory cytokines. These proinflammatory molecules diffuse into the local environment to show their effects throughout the inflammation and tissue repair processes via modulating the neural tissue-related cytokine expression. The locally acting cytokines taking part in the study then pass to the circulation for regulating systematic effects. In our opinion, the alterations on their serum levels might be superficially determine an injury and/or repair process on peripheral nerve. However, the intraneural and intramuscular levels of these cytokines may strongly enhance the significance of the current data. Further investigations involving especially tissue cytokine levels and related molecules contributing to the repair process for I/R

injury might be helpful in figuring out the exact recovery mechanism of the peripheral nerves and to better compare the aforementioned effects on different tissues.

ACKNOWLEDGEMENTS

This study was supported by Research Foundation of Mersin University, Turkey (SBE-TTB (OTB)-2001-3). We wish to thank Derya U. Talas, Assoc. Prof., MD for his excellent support and Arzu Kanik, Assoc. Prof., PhD. for the statistical analysis.

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