Interstitial Adenosine and Serotonin in the Feline Lumbar Spinal Cord during Short-Term Ischaemia

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Summary
During controlled ischaemia (aortal snare occlusion) of the lumbar spinal cord, microcirculatory (laser-Doppler flowmetry) and segmental neurophysiological parameters (monosynaptic reflexes, polysynaptic reflexes, cord dorsum potential = CDP) as well as interstitial concentrations of adenosine and serotonin (5-HT) were determined in the grey matter using the microdialysis/HPLC method. Ischaemic periods of 1–7 min with a residual blood flow in the lumbar spinal cord of 10–30 % of the preischaemic control blood flow caused a blockade of spinal pathways and an increase of concentrations of interstitial adenosine and 5-HT. This increase started immediately after the initiation of the ischaemic period and reached a maximum at the end or shortly after the end of the ischaemic period during postischaemic hyperaemia. A close correlation between the duration of ischaemia and the interstitial concentration of adenosine and 5-HT was not found. Repetition of ischaemic periods in an experiment did not lead to an extracellular accumulation or an exhaustion of the release of 5-HT, whereas some indication was found for an exhaustion of adenosine release. The course of the increase of interstitial adenosine and 5-HT was partly found to correlate to the loss and recovery of the CDP following ischaemia. The concentrations usually reached control levels before spinal reflexes reappeared. The highly dynamic changes in concentrations of adenosine and 5-HT in the extracellular space of the spinal cord during and after short-term ischaemia revealed some relation to the time course of recovery of segmental spinal functions by reflecting the course of spinal neuronal metabolism.

Key words
Spinal cord ischaemia - Microdialysis - Adenosine - Serotonin - Laser-Doppler flowmetry

Introduction

In recent years, evidence has accumulated that ischaemic neurological damage is the final consequence of a cascade of processes that results from an imbalance between excitatory and inhibitory stimuli. The serotonergic system attracted particular interest, since 5-hydroxytryptamine (5-HT) is a potent vasoconstrictor and has both excitatory and inhibitory effects via an action on different receptors in the central nervous system (Mayhan et al. 1988, Prehn et al. 1993). Serotonin antagonists were successfully applied as protective agents for prolongation of ischaemic tolerance of the nervous tissue (Faraci and Heistad 1992, Zivin and Venditto 1984). In contrast, adenosine possesses protective properties itself partly by causing dose-dependent vasodilatation (Gidday et al. 1995, Herold et al. 1994). The most likely source of adenosine is AMP dephosphorylation (Latini et al. 1996).

Microdialysis studies revealed substantial increases of extracellular concentrations of adenosine and 5-HT following cerebral ischaemia (Baker et al. 1991, Benveniste 1989, Globus et al. 1992, Hagberg et al. 1987), but were rare and without significant results for the spinal cord (Rokkas et al. 1995). Nevertheless, such investigations during ischaemia of the spinal cord seem to be an especially useful experimental paradigm because of the uniform spinal morphology, the accessibility to neurophysiological investigations and the relatively equal distribution of the substances in neighbouring segments as was shown for 5-HT (Zivin and Stashak 1983). Therefore, we used a model of spinal ischaemia in cats with ischaemic periods of
controlled duration and depth (registered by laser-Doppler flowmetry; Frerichs and Feuerstein 1990, Lindsberg et al. 1992) and with monitoring of the excitability and activity of spinal neurones (Kolenda et al. 1997).

This model allowed a detailed investigation of the time course of extracellular concentrations of adenosine and 5-HT (using the technique of microdialysis and high performance liquid chromatography at a high time resolution) in correlation to spinal cord blood flow and neurophysiological parameters. Repeated ischaemic periods lasting for 1–7 min have been performed in order to find out, if depleatory or accumulatory effects for the release of adenosine and serotonin may occur.

**Methods**

**General procedures**

The experiments were carried out on 10 adult cats (weight range 2.9 to 4.3 kg). Under ether-halothane-nitrous oxide general anaesthesia the animals were tracheotomised, anaemically decapitated by ligation of the carotid arteries, their branches and the vertebral arteries, spinalized at the C1 level, paralysed with pancuronium bromide (Pancuronium "Organon"; about 0.1–0.2 mg/kg per hour i.v.), and artificially ventilated. After spinalisation the anaesthesia was discontinued. The spinal preparation was used in order to obtain well defined stable spinal reflex conditions without any influence from supraspinal structures or anaesthetic drugs (for details see Kniffki et al. 1981, Kolenda et al. 1997). Arterial blood pressure was monitored in parallel in a common carotid artery and femoral artery. Mean arterial blood pressure was kept between 37.5 and 38.5 °C by extracorporal heating. The spinal cord was exposed from L4 to L7. A positive-pressure ventilated, pCC pressure was kept above 80 mm Hg, if necessary by a catheter from the second common carotid artery, combined with a blood volume reduction (25–60 ml) and thus corresponds to blood flow. In order to determine the degree of ischaemia, the percentage change of flux values was calculated. For that purpose the measured signals were corrected by subtraction of the signal obtained after circulatory arrest representing zero flow (dark noise). The corrected preischaemic records were taken as flow of 100 %. The mean of 100 single values representing 100 seconds was averaged for preischaemic flow calculations and the average out of 60 to 420 values for the 1 to 7 min periods of aortic occlusion was calculated as residual spinal cord blood flow by the formula:

\[
\text{Ischaemic SCBF}_{LD}(\%) = \frac{\text{(IF}_{LD} - \text{DN)} \times 100}{\text{PIF}_{LD} - \text{DN}}
\]

**Laser-Doppler flowmetry (LDF)**

Spinal cord blood flow (SCBF) was measured throughout the experiments by laser-Doppler flowmetry using the MBF3D laser blood flow monitor (Moor Instruments, Axminster, UK; Barnett et al. 1990). The instrument emits a continuous helium-neon laser beam on two channels, with a wave-length of 780–820 nm. The sample volume covers a radius of about 1.5 mm. For registrations the time constant for signal processing was chosen between 0.1 and 0.5 seconds, the processing rate was 10 Hz. Data logging was performed digitally on a PC using a specific data handling software (Moorsoft 4.3). The two laser Doppler probes (model P3) were positioned by micromanipulators to spots having no vessels with a diameter of more than 0.1 mm at a distance of about 1 mm from the lumbar spinal cord (L4–L5). For calculation of the SCBF by LDF (SCBF_LD) surface measurements of the "flux" value were taken, which is calculated from the cell concentration and cell velocity and thus corresponds to blood flow. In order to determine the degree of ischaemia, the percentage change of flux values was calculated. For that purpose the measured signals were corrected by subtraction of the signal obtained after circulatory arrest representing zero flow (dark noise). The corrected preischaemic records were taken as flow of 100 %. The mean of 100 single values representing 100 seconds was averaged for preischaemic flow calculations and the average out of 60 to 420 values for the 1 to 7 min periods of aortic occlusion was calculated as residual spinal cord blood flow by the formula:

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**Neurophysiological recordings**

Mono- and polysynaptic transmission within the spinal cord was tested by recording segmental motor reflexes from lumbar ventral roots L7/S1. Reflexes were elicited by stimulation (single or double rectangular pulses of 0.1 ms duration, recurrence frequency 0.5 Hz, stimulation intensity 5 times threshold strength for muscle nerves and 1.2–1.8 times threshold strength for cutaneous nerves) of nerves to the left posterior biceps semitendinosus (PBSt, flexor), gastrocnemius soleus (GS, extensor) and/or stimulation of cutaneous afferents (sural nerve, Sur; cutaneous branch of superficial peroneal nerve, SPC). The afferent volley as well as the cord dorsum potential (CDP, representing the evoked activity of first order interneurones when stimulating cutaneous nerves) were recorded from the dorsal root entry L7 (Kolenda et al. 1997). All recordings were performed with a bandpass of 10 Hz–10 kHz. The spinal cord and the prepared nerves were covered with warm paraffin oil. The animals were rigidly fixed in a frame.
High performance liquid chromatography (HPLC)

Concentric dialysis probes (probe membrane cylinder 2 x 0.5 mm, pores with molecular cut-off at 20 kD) were inserted into the grey matter of the spinal cord at segment L4 (depth of the tip 2.5 mm). After a stabilisation period of 90 min, the extracellular space of the spinal cord was microdialysed with Ringer's solution at a flow rate of 2 ml/min. Samples were collected every minute (adenosine) or every 2 min (serotonin) and deeply frozen until further analysis. These durations of the collection periods proved to be the detection limits for achieving reliable data with our technique. The measured values were plotted as continuous lines in both figures.

The Applied Biosystems 140B Solvent Delivery System was used for the chromatography of adenosine in conjunction with an Applied Biosystems 785A Programmable Absorbance Detector. Measurements were performed at a wavelength of 254 nm. Chromatographic separation was achieved using a 250 x 1 mm spherisorb ODS2 5 mm reversed-phase column. The mobile phase consisted of 0.01 M sodium phosphate with 15 \% methanol. After adjusting pH to 6.1 with NaOH the mobile phase was filtered through a 0.2 mm regenerated cellulose filter. All separations were performed isocratically at a flow rate of 50 ml/min.

For the chromatography of serotonin (5-HT) the Applied Biosystems 140B Solvent Delivery System was used in conjunction with an ESA Coulochem II detector equipped with a high sensitivity analytical cell (model 5011) containing two electrodes in series. An enhanced response amperometric electrode was coupled with a coulometric electrode in a stainless steel body. During measurements, the first coulometric electrode was set at +200 mV and the second amperometric electrode was set at +250 mV. In order to reduce disturbing white noise a model 5020 guard cell was used consisting of a single porous graphite working electrode set at 300 mV. Chromatographic separation was achieved using a 125 x 2 mm spherisorb ODS2 3 mm reversed-phase column. The mobile phase consisted of 0.01 M sodium phosphate, 0.5 mM EDTA, 0.01 mM 1-octanesulfonic acid and 14 \% methanol. After adjusting pH to 2.5 with phosphoric acid the mobile phase was filtered through a 0.2 mm regenerated cellulose filter. All separations were performed isocratically at a flow rate of 100 ml/min.

Fig. 1. Extracellular concentrations of adenosine and registrations of neurophysiological and local circulatory parameters before, during and after ischaemic periods of 3 - 7 min duration (marked in grey). Traces from the top: amplitude of the monosynaptic reflex from a flexor muscle (PBS\textit{t}), of the cord dorsum potential evoked by SPC stimulation (CDP), the extracellular concentration of adenosine in the grey matter and the relative blood flow in L4 (SCBF\textsubscript{LD}). The monosynaptic reflex of PBS\textit{t} is shown as representative for reflexes. Ischaemias of increasing duration revealed a residual blood flow of 23 \% (7 min) to a maximum of 41 \% SCBF\textsubscript{LD} (4 min) compared to preischaemic control levels. The course of the hyperaemic phase of SCBF\textsubscript{LD} in the first few minutes was partly influenced by the reinfusion of blood that had been taken from the proximal circulation during snare occlusion of the descending aorta in order to reduce collateral circulation.
Results

Basic effects

Snare occlusion of the descending aorta just below the origin of the subclavian artery caused an immediate reduction of lumbar spinal cord blood flow (SCBF_LD; Fig. 1). Monosynaptic reflexes to PBSt and GS and polysynaptic reflexes from cutaneous afferents (data not shown) disappeared after a transitional increase (30–60 s) within the first 2 min of ischaemia (Kolenda et al. 1997). The cord dorsum potential (CDP) dropped progressively down without exhibiting any initial activation and reached zero within about 3 min if the ischaemic period lasted long enough. Extracellular tissue concentrations of adenosine (Fig. 1) as well as of serotonin (5-HT; Fig. 2) started to increase immediately after beginning of the ischaemic period. The calculated levels of SCBF_LD during the ischaemic periods from the onset to the end of aortic occlusion were around 10–30 % compared to the preischaemic control levels. With these SCBF_LD the concentrations of adenosine and 5-HT grew rapidly until the snare occlusion of the aorta was released. Maximum concentrations of both substances were reached at the end or shortly after the end of the ischaemic period and started to decline with full development of the postischaemic hyperaemic period. Due to the duration of the sampling period of one or two minutes a more precise determination of the time course was not possible. Coincidentally with the development of the postischaemic hyperaemia the amplitude of the CDP started to rise. The recovery of mono- and polysynaptic reflexes did not begin until most of the CDP had been re-established and the extracellular concentrations of adenosine and 5-HT had returned to control levels.

Fig. 2. Extracellular concentrations of serotonin (5-HT) and registrations of neurophysiological and local circulatory parameters before, during and after ischaemic periods of 3 and 4 min duration (marked in grey). Parameters as in Fig. 1. Residual blood flow of the three ischaemic periods of 3 min and the one period of 4 min varied between 7 % and 13 % SCBF_LD compared to preischaemic control levels.

Adenosine

The concentration of adenosine in the lumbar spinal cord was analysed in 3 experiments with a total of 20 ischaemic periods of 1–7 min (median 3 min) duration. The depth of ischaemic periods was quite stable with an ischaemic SCBF_LD of 21.7 % (quartiles 16.0 and 28.6 %). The time window for sample collection was 1 min. Ischaemic periods of 1 min duration (n = 3) led to slight increases of the extracellular concentration of adenosine which did not exceed 5 μmol/l. With 2 min of ischaemia or more the concentration reached a maximum of at least 10 μmol/l within about 6–8 min after the onset of ischaemia (Fig.1). The subsequent decline of the concentration to control values was less steep than its rising part. A significant correlation between the duration of the ischaemic period and the duration of increased adenosine levels or its maximum values was not found for the range of investigated ischaemic periods. But it could not be excluded that the duration
of the ischaemic period was one of the factors that influenced the amount of released adenosine, although the highest concentration of 120 \( \mu \)mol/l was observed after an ischaemic period of 2 min duration (data not shown). An influence of the repetition of ischaemic periods was observed in so far as the highest concentrations of adenosine were found during the first ischaemic periods in an experiment, all lasting less than 5 min. A reduced release of adenosine was observed, furthermore, if longer ischaemic periods (more than 4 min) were repeated (Fig. 1). The duration of the increase of extracellular adenosine was not affected by the repetition of ischaemic periods.

With short-term ischaemias, an almost mirror-like behaviour of the extracellular concentration of adenosine was found in comparison to the amplitude of the CDP: the peak concentration was reached coincidentally with complete loss of the CDP and the concentration returned to its control level at the time of complete recovery of the CDP or shortly before.

Serotonin (5-HT)

In 7 experiments with a total of 46 ischaemic periods of 1—6 min duration (median 3 min) the concentration of 5-HT was analysed in the lumbar spinal cord at a time window of 2 min for sample collection. The median ischaemic \( SCBF_{LD} \) was 11.6 % (quartiles 9.0 and 29.9 %). Concentrations of 5-HT ranged between 2 and 200 nmol/l. If the release of 5-HT during ischaemic periods of 3 min (n = 25) was compared to that of longer periods, there was a tendency to a prolonged decay of the 5-HT concentration in the latter cases (Fig. 2). The time course of the increase of extracellular 5-HT was comparable to that of adenosine, with a steep increase and a maximum concentration after the end of the ischaemic period. There were great interindividual differences in the observed maximal concentrations of 5-HT (e.g., up to 200 nmol/l following two ischaemic periods of 4 min duration in one experiment compared to 8 nmol/l in another animal shown in Fig. 2).

No influence of the repetition of ischaemic periods was observed either by the extent or the duration of 5-HT release. Control concentrations of adenosine as well as of 5-HT were generally stable between repeated ischaemic periods. In one extended experiment (14 ischaemic periods of 1—5 min duration), a slight increase of control values occurred after several ischaemic periods followed by a subsequent decrease. The correlation of the time course of 5-HT concentrations to the time course of the CDP was comparable to that found for adenosine. However, no direct correlation between the value of the maximally reached 5-HT concentrations and the time course of the depression of mono- and polysynaptic (data not shown) spinal reflexes was found, similarly as in the case of adenosine.

Discussion

Extracellular microdialysis combined with high performance liquid chromatography allowed dynamic measurement of the increase of interstitial adenosine and serotonin (5-HT) in the lumbar spinal cord during and after ischaemic periods of 1—7 min duration. The time course of increased concentrations of adenosine and 5-HT was relatively constant with an initial steep increase, a peak concentration after the end of the ischaemic period and a subsequent slower decay. Due to the time window of 1 min (adenosine) or 2 min (5-HT) for sample collection by microdialysis, this time course could only be determined roughly. Nevertheless, these time windows allowed for the first time the registration of highly dynamic changes of the extracellular concentrations of adenosine and 5-HT in direct correlation to spinal cord blood flow (\( SCBF_{LD} \)) and to spinal neuronal functions. However, the technique is probably too rough to provide more detailed correlation between the time course of the release of adenosine and 5-HT and the duration and depth of ischaemic periods in the chosen range. More exact correlation was found between the duration and depth of ischaemic periods and the duration of postischaemic depression of spinal cord reflexes and the CDP (Kolenda et al. 1997).

Most of the spinal ischaemic periods revealed an ischaemic \( SCBF_{LD} \) of 10—30 % compared to the preischaemic \( SCBF_{LD} \) and therefore ischaemic periods had to be judged as moderate (\( SCBF_{LD} \) 20—50 %) or deep (\( SCBF_{LD}<20 \% \)) (Kolenda et al. 1997). A fundamental difference of neurophysiological or biochemical reactions between longer or less extensive ischaemic periods was not to be expected in this range. The duration of ischaemia was identified to be a more reliable indicator for the duration and completeness of neurophysiological recovery than the depth at least in the ranges of ischaemia employed (Kolenda et al. 1997, Kolenda et al. in press).

Adenosine is known as an indicator of hypoxic cell metabolism following exhaustion of energetic phosphates and acts as an inhibitory neurotransmitter (Rudolphi et al. 1992). The mechanism of adenosine release is not actually known (Matsumoto et al. 1992). Two mechanisms could be considered in connection with our findings. Either the quantity of extracellular adenosine directly correlates to the extent of the ischaemic insult on the metabolism of spinal neurones, or only a limited quantity of adenosine is released as soon as spinal neurones are exposed to a threshold dose of ischaemia. This latter hypothesis is accord with the findings of Matsumoto et al. (1992) who showed that increases of extracellular adenosine occurred especially if cerebral blood flow in the cat was reduced to 20—25 ml/100 g/min and that the elevation of extracellular adenosine was only transient under continued ischaemia. Furthermore, former
investigations showed that the intracellular concentration of adenosine under ischaemic conditions is 10-times higher than the extracellular concentration (Hagberg et al. 1987).

From our experiments there is an indication that the amount of adenosine release depended on the extent of the ischaemic lesion and furthermore on the content of intracellular adenosine, because its content was exhausted towards the end of the experiment after repeated or longerlasting ischaemic periods. This assumption of exhaustion of intracellular adenosine was supported by the finding shown in Figure 1. After ischaemic periods with increasing duration there was a decrease of the 5-HT release together with a decrease of postischaemic hyperaemia and spinal response amplitudes in comparison to the preischaemic control levels. Increasing numbers of incomplete recoveries of spinal responses have particularly been shown after prolonged ischaemic periods lasting more than 3 min (Kolenda et al. in press).

Serotonin is considered as a complex mediator of impairment caused by ischaemia in the nervous tissue (Harrison and Ellam 1981, Globus et al. 1992). It has been shown to be present in substantial concentrations in the grey and white matter of the spinal cord (Zivin and Stashak 1983). In complete tissue samples of the spinal cord, Zivin and Stashak (1983) found an increase of 5-HT after ischaemic periods lasting for 5 min and a sustained decrease after ischaemic periods of 14 and 20 min duration. Postischaemic increases of extracellular concentrations of 5-HT up to 30 nmol/l have been found by microdialysis in the caudate nucleus of rabbits with a delay of 5 min following 5, 10 and 15 min of cerebral ischaemia (Baker et al. 1991). Similar as in our experiments, no strict correlation was found between the peak level and duration of 5-HT release and the duration of cerebral ischaemia but, in contrast to cerebral ischaemia, after spinal cord ischaemia no comparable delay of the increase of 5-HT was observed. Some relation between an increasing duration of ischaemia and a prolongation of increased 5-HT concentration could partly occur (Fig. 2). Repetition of short-term spinal ischaemic periods in an experiment was generally possible without particular influence on the amount and time course of neurophysiological and spinal cord blood flow parameters during and after ischaemia, as long as the intervals were kept long enough to allow complete recovery between the ischaemic periods (Kolenda et al. 1997). Similarly, there was in general no effect of repeated ischaemic periods on the behaviour of extracellular 5-HT concentrations. The effect of repeated ischaemic periods on nervous tissue has also been studied by Decombe et al. (1993) who found comparable concentrations of extracellular 5-HT after two ischaemic periods of 20 min duration in the rat striatum.

Adenosine as well as 5-HT have been extensively investigated for their vascular effects. Adenosine produces dose-related dilatation of arteries while 5-HT is a potent dose-dependent vasoconstrictor in the central nervous system (Mayhan et al. 1988). For a 20 %-change in the diameter of cerebral arteries, topical application of 0.1 mmol adenosine and of 0.1 μmol serotonin were found to be necessary. These effects were preserved after 10 min of ischaemia. In the rat spinal cord an increase of SCBF₁D was found after local application of 0.01–0.1 μmol adenosine whereas below a dose of 0.001 μmol adenosine no effect was registered (Karlsten et al. 1992). Therefore, alterations of SCBF₁D were not likely to appear in consequence of concentration changes of adenosine or 5-HT following short-term ischaemia in our experiments, even when the wide range of concentration levels seen in different experiments are taken into account. This wide range might not only be due to different individual concentrations but might also be based on the fact that due to the small time window, the biochemical analysis was performed near the detection limit. However, the time course of concentrations and its interpretation was not affected by interindividual differences. The beginning of the hyperaemic periods coincided with the maximum concentration of adenosine so that the effect of adenosine cannot be excluded. But according to the further course of adenosine concentrations and hyperaemia, adenosine cannot be the only responsible factor.

The time course of extracellular concentrations of adenosine as well as of 5-HT during and after ischaemia showed a mirror-like behaviour to the time course of the CDP. The CDP which can be taken as an indicator of the activation of first-order spinal interneurones to peripheral stimulation, was found to be a better indicator for the status of the spinal cord than the reflex responses which are more sensitive to ischaemia and are therefore less graded (Kolenda et al. 1997). The delay from the end of the ischaemic period to the beginning of reflex recovery was assumed to indicate a depression of synaptic transmission and possibly of motoneuronal synaptic excitability. Evidently this period ended at the same time when the concentrations of adenosine and 5-HT had returned to control values. Coincidentally, the oxygen concentration in the gray matter of the spinal cord which increased far above control values after the ischaemic periods and which has been looked upon as a resultant of supply (SCBF₁D) and consumption (neuronal activity) started to decrease (Kolenda, Steffens, Nagel, Schomburg, unpublished observation). This decline of postischaemic hyperoxia was assumed to be due to increasing oxygen consumption caused by the onset of neuronal recovery which is indicated by the recovery of reflex activity. The postischaemic normalization of the extracellular concentrations of
adenosine and 5-HT may indicate the recovery of neuronal metabolism in a similar way.  

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References


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