Cerebral Platelet Thromboembolism and Thromboxane Synthetase Inhibition

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SUMMARY Platelet aggregating sodium arachidonate was slowly infused into the internal carotid artery (1 mg, 100 μl, 1 μl/s) of nitrous oxide anesthetized rats. The electroencephalographic activity recorded by a Cerebral Function Monitor from the injected hemisphere was reduced within minutes. The somatosensory evoked responses to contralateral electrical stimulation of the whisker area were eliminated on the same side in most cases when measured five and fifteen minutes after the infusion. The brain was frozen in situ with liquid nitrogen after fifteen minutes. Regional tissue analysis showed ipsilateral derangement of the cerebral energy state and increased lactate levels. Pretreatment with the platelet antiaggregating thromboxane synthetase inhibitor OKY-1581 (Sodium-3-4-(3-pyridylmethyl)phenyl-2-methyl-acrylate), 30 mg/kg i.v., fifteen minutes before the sodium arachidonate infusion prevented cerebral energy failure and elimination of the sensory evoked responses.

PLATELET AND COAGULATION FUNCTION in relation to thromboembolic stroke is far from fully elucidated.1 The preventive treatment of cerebral thromboembolism is a matter of debate. No entirely satisfactory link has emerged between present pharmacological knowledge and clinical application of platelet antiaggregatory drugs.2 Most therapeutic approaches are based on the assumption that the homeostasis between platelet thromboxane, TXA2,3 and vascular prostacyclin PGI2,4 is essential for the hemostatic balance.

One way of interfering with the formation of platelet aggregating and vasoconstricting TXA2, is by cyclo-oxygenase inhibition, another by thromboxane synthetase inhibition.2, 5 The cyclo-oxygenase inhibitors block the formation of vascular PGI2 as well as platelet TXA2. It has been proposed that thromboxane synthetase inhibitors, besides more selectively limiting the formation of TXA2, could in vivo divert the accumulated proaggregatory endoperoxides towards increased formation of platelet antiaggregating and vasodilating PGD2 and PGI2, i.e. enhance the negative feedback mechanism.5, 6

Platelet aggregates have previously been observed in cortical surface arteries and in intracerebral precapillary arterioles after infusion of platelet aggregating sodium arachidonate into the carotid artery of rabbits and rats.7-9 The cerebral blood flow and the electroencephalographic activity was reduced. Pretreatment of the heparinized rats with aspirin, i.e. a cyclo-oxygenase inhibitor, strongly reduced arachidonate-induced platelet aggregation in vitro but offered little protection against arachidonate-induced stroke.4

The purpose of our study was to investigate if the thromboxane synthetase inhibitor OKY-1581 prevented cerebral transmission and energy failure induced by an intracarotid infusion of sodium arachidonate, which provokes cerebral thromboembolism and cerebrovascular occlusions-flow reduction in unpretreated rats. The previously described experimental model was slightly modified — sodium arachidonate was infused into the internal carotid artery of non-heparinized rats to avoid any confounding influence from an anti-clotting agent.

Material and Methods

Animals

The experiments were performed on male Wistar rats (SFP strain, Møllegaards Avislaboratorium) weighing between 320 and 380 g. The animals had free access to tap water and food pellets until operation.

Chemicals

A 33 mM solution of sodium arachidonate (Chemicon) in 50 mM sodium carbonate in saline was prepared as described by Furlow and Bass,8 stored at −50°C to −70°C and warmed to +23°C immediately before usage. OKY-1581 (Sodium-3-4-(3-pyridylmethyl)phenyl-2-methylacrylate) kindly provided by Kissei Pharmaceutical Co Ltd was dissolved in Krebs-Hensleit solution to a concentration of 30 mg/ml. Heparin (Vitrum) was diluted to a concentration of 300 IU/ml by Krebs-Hensleit solution.

Platelet Aggregation

The platelet aggregating activity of the sodium arachidonate solution and the interference by heparin and OKY-1581 on aggregate formation was checked on platelet rich plasma from a separate group of rats in an aggregometer according to Born10 as modified by Thorngren and Gustavsson.11 Nine ml of blood was mixed with one ml of 3.8% trisodium citrate in siliconized glass tubes and centrifuged at 260 G for 10 minutes at room temperature immediately after collection (Centrifuge Hettich EBA 3 S). The platelet rich plasma was separated and after further centrifuging at 2600 G for 20 minutes the platelet poor plasma was collected and later used as reference. The platelets were counted...
in a Bürker chamber and the platelet rich plasma was
diluted with platelet poor plasma to a count of 200,000
platelets per μl. The aggregation was measured in a
Payton aggregation module (Model 600) as maximal
velocity of the increment in light transmission per time
unit (Vmax).

Operative Technique
The animals were initially anesthetized with 3%
halothane and thereafter with 1% in N2/O3 (70:30)
via a face mask. Temperature was kept close to 37.5°C
by external heating. For the selective and non-occlusive
infusion of sodium arachidonate or saline into the
internal carotid artery the pterygopalatine artery was
ligated and the right external carotid artery was cannulated
in retrograde direction leaving the tip of the catheter
1–2 mm from the carotid bifurcation (fig. 1). A
femoral artery was cannulated to obtain continuous
recording electromanometrically (Elema) of mean artes-
trial blood pressure (MAP) and for blood sampling. A
femoral vein was cannulated for the i.v. administration
of chemicals. The animals were tracheotomized and
connected to a Starling type respirator delivering 1%
halothane in N2/O3 (70:30). Immobilization was
accomplished by d-tubocurarine (Vitrum) 1.7 mg/kg.
Four goldplated copper bolts were inserted into the
skull bone in fixed bilateral frontal and parietal posi-
tions for electrophysiological recording. Two needle
electrodes were positioned 5–7 mm apart subcuta-
aneously in the whisker areas for electrical stimulation.
Thereafter the halothane but not the nitrous oxide sup-
ply was terminated. One hour later the experiment was
performed after arterial blood samples had been ana-
lyzed for blood gases, pH, blood glucose and hemato-
crit. The animals were replenished with fluid for losses
incurred throughout the operative procedure. An intra-
arterial injection of Krebs-Hensleit solution (50 μl)
was invariably done in all animals before the main
intracarotid infusion. If the electrophysiological pa-
rameters were affected by this injection — presumably
due to the release of a preformed thrombus at the tip of
the indwelling catheter — the animal was excluded
(n.2).

Electrophysiological Techniques
The electroencephalogram (EEG) was continuously
recorded separately from each hemisphere on an
Elema Mingograph. The bipolar frontoparietal lead on
the injected side was fed into a Cerebral Function
Monitor (CFM, Devices Ltd) plotting the EEG through
a frequency selective filter (2-20 Hz). CFM provides
greater amplification with increasing wave frequency
measuring quantitatively changes in the amplitude of
the EEG. Somatosensory evoked responses (SER)
were recorded contralateral to electrical stimulation of
the whisker area as previously described by Agardh et
al. The amplitude of the SER was measured from the
initial positive to the following negative peak (parietal
electrode) and the latency was measured to each of
these two peaks. The SER was recorded before and
after any injection into the carotid artery.

Experimental Groups
The experiments were performed while the animals
were under continuous electroencephalographic recor-
ding. Fifteen minutes after a slow infusion of 1 mg
sodium arachidonate (1 mg, 100 μl, 1 μl/s) into the
right carotid artery the brain was frozen in situ14 (n.9).
Another experimental group was pretreated with
OKY-1581, 30 mg/kg i.v., fifteen minutes before the
sodium arachidonate injection (n.6). In an unpretreat-
ed control group (n.4) equal amount of Krebs-Hensleit
solution (100 μl) was infused into the right carotid
artery instead of sodium arachidonate. In an additional
control group (n.3) no intracarotid injection was made
before the brain was frozen. Due to technical difficul-
ties, i.e. splitting of the brain when chiselled out of the
skull bone, two animals were excluded from analysis
of energy metabolites.

Brain Tissue Analysis
The frozen brain was chiselled out at liquid nitrogen
temperature and stored at −80°C until analysis of en-
ergy metabolites. Tissue samples from the lateral part
of the parietal cortex and from the caudate-putamen
were dissected and weighed separately at − 20°C. The
tissue was extracted at this temperature with HCl-
methanol and subsequently with perchloric acid at
0°C. The extracts were neutralized with a KOH-imida-
zole base-KCl mixture as previously described. The
contents of phosphocreatine (PCr), ATP, ADP,
AMP, lactate, pyruvate and glucose were analyzed by
enzymatic fluorometric techniques. For details of the
procedure see Folbergrová et al15–16 and Lowry and
Passonneau. The energy charge of the adenine nucleotide pool
(EC) was calculated according to Atkinsson.18 Statistical
analysis were performed with Fisher's exact prob-
ability test, Mann-Whitney U-test and analysis of vari-
ance (ANOVA). Values are given as mean ± standard
deviation (SD).

![Diagram](https://example.com/diagram.png)

**FIGURE 1.** The non-occlusive carotid infusion technique
through a retrograde cannula implanted into the external carotid
artery leaving the tip of the catheter close to the carotid bifurcation.
The pterygopalatine artery was ligated to minimize extracerebral passage of the injected substance.
Results

Platelet Aggregation

Platelet aggregation was provoked within seconds by a minimal concentration of 170 μg sodium arachidonate per ml platelet rich plasma. Pretreatment of the rats with heparin (n=2), 270 IU/kg i.v., augmented the platelet aggregating effect of 3.3 mM sodium arachidonate (V max = 6%/s) while pretreatment with OKY-1581 (n=2), 30 mg/kg i.v., rendered the platelets less sensitive to arachidonate (V max = 0.1%/s) compared to nonpretreated rats (V max 1%/s) (fig. 2).

Physiological Parameters

Body temperature, arterial blood gases, pH, blood glucose, hematocrit and blood pressure measured before any intracarotid infusion was made did not differ significantly among the groups (analysis of variance, 95% probability level). MAP decreased transiently from 156 ± 14 to 129 ± 20 mm Hg in the sodium arachidonate injected animals. In the OKY-1581 pretreated rats MAP decreased from 152 ± 17 to 128 ± 21 mm Hg after the sodium arachidonate administration. In rats infused with Krebs-Hensleit solution MAP was unaffected by the intracarotid injection.

Neural Transmission

The SER was eliminated on the injected side in seven out of nine animals when recorded five and fifteen minutes after the infusion of sodium arachidonate (p < 0.01 compared to the non-injected unaffected hemisphere, Fisher’s exact probability test). A transient decrease of the SER-amplitude was seen in one rat five minutes after the infusion. In another rat the SER-amplitude was unaffected. The SER-elimination was accompanied by a reduction of the CFM-amplitude ipsilaterally sometimes preceded by a short increase. The EEG showed varying abnormality with episodic delta waves contralaterally or bilaterally and/or ipsilateral amplitude depression (fig. 3). SER-elimination or depression of the CFM-amplitude was not seen in rats (n=4) infused with equal amount of saline (p < 0.05 compared to the sodium arachidonate injected group) or in rats without any intracarotid infusion (n=3). Rats (n=6) pretreated with OKY-1581 before the sodium arachidonate infusion did not show SER-elimination (p < 0.01 compared to the nonpretreated sodium arachidonate group). Transient decrease of the SER-amplitude was seen in three rats. The rat showing the most marked effect on SER is shown in figure 4. In the three other rats the SER-amplitude was entirely unaffected.

Cerebral Energy Metabolites

The values of the tissue energy charge and lactate in the parietal cortex and the caudate-putamen of both hemispheres are shown in figure 5. The controls are presented as one group in the figure. In the parietal cortex of the injected hemispheres of the non-pretreated sodium arachidonate infused animals, the energy charge was significantly decreased and the lactate concentration significantly increased compared to the contralateral hemisphere. The lactate level was significantly increased in the caudate-putamen. In the parietal cortex and the caudate-putamen of the noninjected hemispheres the values were similar to those of controls. No interhemispherical differences in energy charge, concentrations of labile phosphates, lactate or pyruvate was observed in the parietal cortex of rats pretreated with OKY-1581 and the values were similar to those of the controls. In the caudate-putamen a small but significant increase in lactate concentration was noted on the injected side (Mann-Whitney U-test).

Discussion

The endothelium forms a non-reactive interface between the artery wall and blood elements such as the platelets. The thrombo-resistance of the endothelium is due to i.a. synthesis of PGI2 and the presence of a heparinlike proteoglucan which combines with antithrombin III adsorbed to the surface. After endothelial injury platelets adhere to exposed subintimal tissue such as collagen and aggregates are formed. Vasoactive, platelet aggregating and proclotting substances are released from the platelets in a potent secretory cascade promoting thrombosis and hemostasis.

In experimental cerebral platelet thromboembolism a hemostatic imbalance is created by endothelial damage and/or local accumulation of platelet aggregating agents. A wide variety of substances other than collagen change the shape of the platelet from a smooth surfaced disc to a sphere with pseudopodia facilitating platelet aggregation. One such substance is arachidonic acid acylated to phospholipids in the cell mem-
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FIGURE 3. The effect of intracarotid sodium arachidonate (NaA) on neural transmission recorded by a Cerebral Function Monitor (CFM) from the injected hemisphere and somatosensory evoked responses (SER, EP-test) and EEG separately from each hemisphere. The infusion of Krebs-Hensleit solution (NaCl) did not affect the amplitude of CFM, SER or EEG. In the majority of rats the infusion of 1 mg NaA was followed within minutes by ipsilateral elimination of the SER and reduction of the CFM amplitude persisting until freezing of the brain in situ fifteen minutes later.

branes. An excessive release of arachidonic acid might in addition to platelet aggregation induce endothelial damage and dysfunction.24-26 Thus, by infusing sodium arachidonate into the carotid artery the homeostasis between vascular PG12 and platelet TXA2 is disturbed promoting platelet aggregate formation23 due to excessive TXA2.

The activation pathways for platelet aggregation include a complex interrelationship between arachidonic acid metabolites, cyclic nucleotides and calcium within the platelets.5, 23, 27 Arachidonic acid can be oxidized by the enzyme cyclo-oxygenase to form endoperoxides. The endoperoxides serve as precursors for the synthesis of the prostaglandins PGD2, PGE2 and PGF2α but are in the platelet mainly converted to the highly unstable intermediate TXA2 by a thromboxane synthetase.3 TXA2 is a powerful stimulant of vascular smooth muscle and a potent inducer of platelet aggregation.

The formation of platelet PGD2 and vascular PG12 from the endoperoxides seems to be an important negative feedback mechanism limiting growth of platelet aggregates and vasoconstriction.

Besides decreasing the formation of TXA2, thromboxane synthetase inhibitors might augment the negative feedback mechanism limiting the growth of platelet thrombi as stated in the introduction. Heparin has been proposed to attenuate this feedback mechanism and increase the formation of TXA2.28,29 Opposing effects of OKY-1581 and heparin on arachidonate induced platelet aggregation was shown in the present study (fig. 2). Heparin should, however, by binding to antithrombin III and increasing the rate at which this protein inhibits the clotting enzymes, decrease the formation of fibrin strands and the consolidation of platelet aggregates. Because of this and the complex interaction between platelet aggregation and clotting,30

FIGURE 4. The effect of 1 mg intracarotid sodium arachidonate (NaA) on neural transmission, recorded as EEG and somatosensory evoked responses (SER, EP-test) from the injected (right) hemisphere and SER from the contralateral hemisphere after pretreatment with OKY-1581 (30 mg/kg i.v.). The EEG and the SER were not affected by injection of OKY-1581 or by intracarotid infusion of NaCl. NaA provoked a transient decrease of SER and a slight increase of slow waves in the EEG after 5 min on the injected side. After 15 min SER and EEG were normalized.
non-heparinized rats were used to test the prophylactic effect of pretreatment with the thromboxane synthetase inhibitor OKY-1581.

The intracarotid infusion of sodium arachidonate provoke a severe reduction of cerebral blood flow within the injected hemisphere, as previously shown with the 14C-antipyrine autoradiographic technique. In some instances small areas of oligemia were found in the territory of the contralateral anterior cerebral artery but the main blood flow reduction occurred within the ipsilateral hemisphere. When injecting small amounts of arachidonate (0.35 mg/kg during 15 sec.) the cerebral circulation was gradually restored after 15 and 45 minutes respectively. We have measured cerebral blood flow with the 14C-iodoantipyrine autoradiographic technique in a few rats with ipsilateral SER-elimination (n=1) and 15 (n=4) minutes after the intracarotid infusion of sodium arachidonate (1 mg, 100 μl, 1 μl/s). The distribution of cerebral blood flow reduction confirmed previous findings, the degree of oligemia varied considerably between animals and regions (unpublished observations).

The existence of two critical thresholds of cerebral blood flow has been recognized, one for the abolishment of synaptic transmission (transmission failure) and the other, somewhat lower, for the derangement of membrane ion potentials and energy metabolism (membrane failure). Because heparinization of the animals is necessary for the proper performance of the 14C-iodoantipyrine technique we choose to test the protective effect of the thromboxane synthetase inhibitor OKY-1581 on SER and cerebral energy state using non-heparinized rats. We wanted to see if OKY-1581 preserved the cerebral blood flow above both the threshold for transmission failure and for energy failure when tested without the confounding influence of an anticoagulant agent.

The intracarotid infusion of platelet aggregating sodium arachidonate (1 mg) provoked ipsilateral elimination of evoked potentials, degradation of labile phosphates and an increase in lactate in the parietal cortex. The heterogeneity and variability was reflected by the fact that the energy metabolites of the caudate-putamen in spite of marked interhemispherical differences of the mean values reached statistical significance only for lactate. Pretreatment of the rats with

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**Figure 5.** The energy charge (figure above) and the lactate level (figure below) in the parietal cortex and the caudate-putamen of controls and fifteen minutes after the intracarotid infusion of 1 mg sodium arachidonate (NaA) with and without pretreatment with the thromboxane synthetase inhibitor (OKY-1581), 30 mg/kg i.v. Injected hemisphere (closed circles). Non-injected hemisphere (open circles). (Mann-Whitney U-test).
platelet antiaggregating OKY-1581 (30 mg/kg i.v.) led to preservation of evoked responses and normal steady state levels of labile phosphates following the arachidonate injection. Thus the thromboxane synthetase inhibitor OKY-1581 in the present experimental model prevented both transmission and membrane failure induced by sodium arachidonate. The discrepancies between previous in vivo studies regarding prevention of cerebral platelet thromboembolism by pretreatment with OKY-1581 may be explained by differences in dosages, animal species and experimental techniques used. OKY-1581, in addition to its thromboxane synthetase inhibiting properties at concentrations >10^{-4} M also inhibits prostacyclin synthetase at concentrations >10^{-6} M in vitro. Furthermore, there are alternative platelet activation pathways not including TXA{subscript}2 for thromboembolism by pretreatment with OKY-1581 (30 mg/kg i.v.) led to preservation of evoked responses and normal steady state levels of labile phosphates following the arachidonate injection.

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