Ischemia Reperfusion Injury in the Isolated Hemoperfused Bovine Uterus – a Model for the Investigation of Anti-Inflammatory Substances?

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Summary
The inflammation model of the isolated hemoperfused bovine uterus was used to introduce a new in vitro model for the investigation of anti-inflammatory substances.
As previous studies demonstrated both an increase in PGE_2 synthesis and an up-regulation of COX-2 and iNOS mRNA by ischemia-reperfusion injury in the model (Braun and Kietzmann, 2004), inhibitory effects of the glucocorticoid dexamethasone, the NSAID flunixin and the selective COX-2 inhibitor DFU (5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)-phenyl-2-(5H)-furanone) were studied. All substances caused a significant decrease in tissue PGE_2 production, while none induced down-regulation of COX-2 mRNA. A slight decrease in the mRNA level of iNOS was observed after 300 minutes of perfusion with dexamethasone-supplemented perfusion medium.
In conclusion, the suitability of the isolated hemoperfused bovine uterus for the investigation of anti-inflammatory substances, especially regarding their COX-2 selectivity, was demonstrated. Use of the isolated hemoperfused bovine uterus in pharmacological research and drug screening may contribute to a reduction of animal testing.

Zusammenfassung: Ischämische Reperfusions-Schäden im isoliert hämoperfundierten Rinderuterus – ein Modell zur Untersuchung entzündungshemmender Substanzen?
Das Entzündungsmodell des isoliert hämoperfundierten Rinderuterus wird als ein neues in vitro Modell zur Prüfung von anti-entzündlichen Substanzen vorgestellt.
Nachdem in vorherigen Studien sowohl eine gesteigerte PGE_2-Synthese als auch eine Hochregulation von COX-2- und iNOS-mRNA durch ischämischen Reperfusionsschaden im hämoperfundierten Myometrium gezeigt wurden (Braun und Kietzmann, 2004), sollten inhibitorische Wirkungen des Glucocorticoids Dexamethason, des NSAID Flunixin und des selektiven COX-2-Inhibitors DFU (5,5-Dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)-phenyl-2-(5H)-furanon) untersucht werden. Alle Testsubstanzen führten zu einem signifikanten Abfall der PGE_2-Produktion im Gewebe, während kein Einfluss auf die Regulation der COX-2 auf mRNA-Ebene zu verzeichnen war. Nach fünfstündiger Perfusion mit Dexamethason war eine leichte Reduktion der iNOS-mRNA vorhanden.
Es wurde gezeigt, dass der isoliert hämoperfundierte Rinderuterus zur Prüfung von anti-entzündlichen Substanzen eingesetzt werden kann und des Weiteren auch geeignet ist, eine Aussage hinsichtlich der COX-Selektivität von Testsubstanzen zu treffen. Durch den Einsatz des Modells in der pharmakologischen Forschung und dem Arzneimittelscreening kann der isoliert hämoperfundierte Rinderuterus einen Beitrag zur Reduzierung von Tierversuchen leisten.

Keywords: bovine uterus, ischemia-reperfusion injury, inflammation, cyclooxygenase, hemoperfusion

1 Introduction

The isolated, tyrode perfused bovine uterus was developed for studies on the mucosal irritation potential of intrauterinely administered antiseptic solutions (Bäumer et al., 2002). Following previous studies on dermal inflammation in the isolated perfused bovine udder (Bäumer and Kietzmann, 2000), the isolated hemoperfused bovine uterus was shown to be a suitable *in vitro* model for acute inflammation induced by ischemia reperfusion injury (Braun and Kietzmann, 2004). Since the manifestation of cyclooxygenase (COX)-2-dependent acute inflammatory reactions has been demonstrated, we investigated whether the isolated hemoperfused bovine uterus is suitable to examine the COX selectivity of test compounds *in vitro*.

The enzyme cyclooxygenase is a key enzyme in the metabolism of arachidonic acid (AA) to prostaglandins (PG) (Hamberg and Samuelsson, 1967). The isoform COX-2 is induced by various factors, e.g. growth factors and pro-inflammatory cytokines (Masferrer et al., 1990; Fu et al., 1990; Xie et al., 1991). While the constitutively expressed isoform COX-1 is mainly responsible for the production of physiological amounts of prostaglandins, inducible COX-2

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plays an important role in various inflammatory diseases (Herschman, 1996; Hinz and Brune, 2002). One of these diseases is ischemia-reperfusion (I/R) injury, which is characterised by an inflammatory reaction in the postischemic tissue, mainly induced by extensive production of oxygen radicals at the onset of reperfusion (Carden and Granger, 2000; Chan, 2002).

As representatives of different classes of antiphlogistic drugs, dexamethasone (a corticosteroid), flunixin (a non-steroidal anti-inflammatory drug, NSAID) and 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)phenyl-2(5H)-furanone (DFU, a selective COX-2 inhibitor) were added to the perfusion medium to simulate systemic application. Their effects on both COX isoforms were determined by measurement of tissue PGE_2 concentration in untreated and locally AA (0.1 or 5 mg/deposit) and arachidonylethanolamide (AEA, 0.1 mg/deposit) treated myometrium. As AEA is exclusively and lower doses of AA are predominantly converted by COX-2 (Yu et al., 1997; Shitashige et al., 1998; Kozak et al., 2001), they are suited for selective determination of COX-2 activity. A higher amount of AA (5 mg) was used to acquire the activity of both isoforms. Further on, the regulation of both cyclooxygenases as well as that of the inducible NO synthase (iNOS) was determined on mRNA level by RT-PCR. Western Blot analysis was used to determine the amount of COX-2 protein in tissue samples of control and dexamethasone-perfused organs.

2 Animals, materials and methods

2.1 The isolated hemoperfused bovine uterus

Uteri were obtained at a slaughterhouse from healthy Holstein Friesian cows older than two years, i.e. after at least one parturition. Ten to fifteen minutes after blood withdrawal the organs were infused with 300 ml of heparinised (120 IU/ml) tyrode solution via the uterine arteries. Only uteri without macroscopic or palpatoric signs of inflammation or pregnancy or with an ovary bearing a distinct *corpus luteum* were used. For hemoperfusion, ten litres of homologous blood were obtained directly at slaughter and stabilised with heparin (18 IU/ml).

After transport to the laboratory, the uterine arteries were connected with the perfusion medium supply via silicon tubes. Venous drainage was established by cannulating the ovaric veins. The organs were transferred into a water bath containing tyrode solution at 39°C. A perfusion flow of one litre per uterus horn and hour was maintained by the use of a peristaltic pump (type 103, Ole Dich, Hividovre, Denmark). The organs were perfused with tyrode solution alone for 30 minutes to rinse blood residues from the vessels before changing the perfusion medium to a mixture of oxygenated blood (80%) and tyrode (20%). Gentle oxygenation of the blood was provided by a dialysis system. This system consisted of a tyrode reservoir (8 l), from which oxygen-saturated tyrode solution was transported via a centrifugal pump (type 2013, EHEIM, Deizisau, Germany) at a flow rate of 500 ml/min (measured with a Flo-Meter 111, McMilan, Pittsburgh, PA, USA) through a hollow fibre dialyser (Diacap SMC 1.0 SD, Braun AG, Melsungen, Germany). To keep the pH in the range of 7.36 to 7.44, pure oxygen was used for oxygenation. The dialysis fluid flowed against the flow of the perfusion medium, so that optimal oxygenation could be achieved. The experiments started after equilibration of hemoperfusion for 15 minutes. Figure 1 gives a schematic overview of the perfusion device. Organ viability was ensured by monitoring glucose consumption, lactate production and the molar lactate/glucose ratio as described previously (Mertens, 2001; Braun, 2002; Braun and Kietzmann, 2004).

In the experiments performed with antiphlogistic substance perfusion, disodium dexamethasone dihydrogenphosphate (Dexa 4 mg inject, Jenapharm, Jena, Germany), flunixin meglumine (Finadyne® RP, Essex Tierarznei, Munich, Germany) or DFU (Merck, Rahaway, NJ, USA) were added to the blood/tyrode mixture. The final concentrations were in accordance with mean plasma levels achieved in cattle after systemic treatment, i.e. 0.1 µg/ml for dexamethasone, 1 µg/ml for flunixin and 0.36 µg/ml for DFU (Braun, 2002). To avoid clearance of the drugs in the dialysis system, the dialysis medium was supplemented with the same concentration of the respective compound.

2.2 Conditions of ischemia-reperfusion

Warm ischemia in the uteri was maintained for 75 minutes, starting with blood withdrawal at the slaughterhouse and ending with the onset of perfusion at the

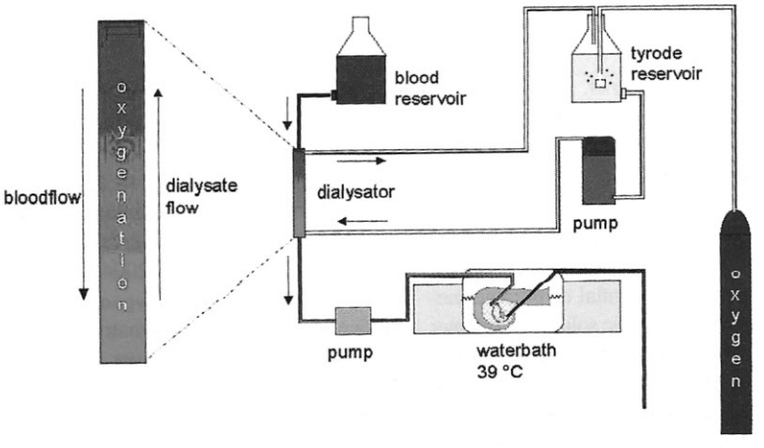

Fig. 1: Schematic overview of the perfusion device.

laboratory. The overall (re-) perfusion time was 345 minutes, including 45 minutes of equilibration and 300 minutes of hemoperfusion *per se*. Figure 2 shows a scheme of the I/R timetable.

2.3 Induction of PGE_2 synthesis in myometrial tissue

Arachidonic acid (AA) and arachidonylethanolamide (AEA) were purchased from Sigma-Aldrich (Steinheim, Germany) and dissolved in pure ethanol. The stock solutions of AA and AEA were diluted in tyrode to achieve final concentrations of 25 and 0.5 mg/ml for AA and 0.5 mg/ml for AEA, respectively. Small deposits (200 µl) of these solutions were injected into the myometrium. According to previously described data (Bäumer et al., 2002), AA (5 mg/deposit) was injected at the start of the experiments, while AEA and AA (0.1 mg/deposit) were injected 60 minutes before tissue biopsy to avoid total degradation of the substrates before sampling. For each biopsy one deposit was set and the tissue samples were taken directly at the site of the deposit. The final concentration of injected ethanol was 10%. 180 and 300 minutes after the beginning of the experiments, biopsies of 6 mm diameter were taken from treated and untreated myometrium and frozen at -196°C.

2.4 Determination of PGE_2 concentration in tissue biopsies

The biopsies were homogenised in phosphate buffered saline and spun for ten minutes at 20000 x g and 4°C. After centrifugation the supernatant was diluted (1:5) and the PGE_2 concentration was measured by ELISA (R&D Systems, Wiesbaden, Germany) according to the manufacturer's protocol. The determined PGE_2 concentrations were related to the amount of protein in the samples, measured with the method of Lowry et al. (1951).

2.5 Isolation of total RNA and protein

Biopsies of myometrial tissue taken directly before and 180 and 300 minutes after starting hemoperfusion were homogenised by grinding in liquid nitrogen. The resulting powder was lysed in 1.5 ml peqGold TRIFAST™ (Peqlab, Erlangen, Germany) and total RNA was isolated and purified with peqGold OptiPure™ (Peqlab, Erlangen, Germany) according to the manufacturer's protocol. Following precipitation, RNA was dissolved in 50 µl RNase-free water.

Protein was isolated from the phenolic residue of the RNA isolation, according to the manufacturer's protocol. Following precipitation, protein was resuspended in 200 µl sodium dodecyl sulphate (SDS, 1%). An aliquot of the solution was used for protein quantification (using Bio-Rad Protein-Assay, Bio-Rad Laboratories GmbH, München, Germany) and the samples were adjusted to the same protein concentration before analysis.

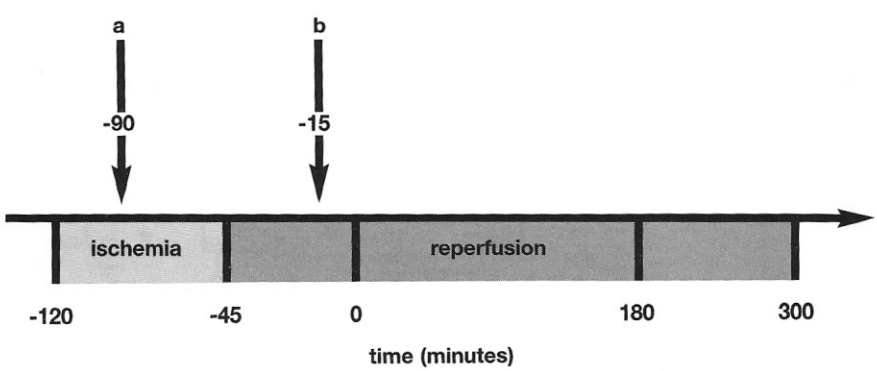

Fig. 2: Timetable of experimental procedure: -120 min: blood withdrawal, -45 min: start of reperfusion with tyrode; 300 min: end of experiment; sampling after 180 and 300 min; a: rinsing of the blood vessels at the slaughterhouse (-90 min); b: start of hemoperfusion (-15 min).

2.6 RT-PCR analysis

RT-PCR analysis was performed using Superscript™ One-Step RT-PCR with Platinum® Taq (Invitrogen, Karlsruhe, Germany). For standardisation of the RNA amount, GAPDH (glyceraldehyde-3-phosphate dehydrogenase) expression was determined using the following primer sequences: upstream, 5'-CCT TCA TTG ACC TTC ACT ACA TGG TCT A-3' and downstream, 5'-GCT GTA GCC AAA TTC ATT GTC GTT ACC A-3'. The expected length of the transcript was 855 bp. RT-PCR was performed over 25 cycles with an annealing temperature of 60°C. The COX-1 primer sequences were as follows: upstream, 5'-AGC CAT ATC GTG GCG TAG ACC-3' and downstream, 5'-CCT CCC ACC TAC AAC GTA GCG-3'. The expected length of the transcript was 538 bp. RT-PCR was performed over 35 cycles with an annealing temperature of 58°C. Since the COX-1 is only regulated under special circumstances (e.g. differentiation processes) (Herschman, 1996) the amplification of COX-1 mRNA serves as an additional parameter for standardisation. The COX-2 primer sequences were as follows: upstream, 5'-GTG GGA GGA TAC ATC TCT CCA TTA ATC-3' and downstream, 5'-CAA GAC AGA TTT TGA ACG AGG ACC-3'. The expected length of the transcript was 164 bp. RT-PCR was performed over 22 cycles with an annealing temperature of 61°C. The iNOS primer sequences were as follows: upstream, 5'-TCC TCC ACC TGT TCC TCG TTC-3' and downstream, 5'-TCT ATG TTC AAG ACA TCC TGC G-3'. The expected length of the transcript was 174 bp. RT-PCR was performed over 30 cycles with an annealing temperature of 58°C.

The RT-PCR products were separated on 1.5% agarose gels and stained with ethidium bromide. Visualisation of the bands was performed with an UV-Transilluminator (Biometra, Göttingen, Germany).

2.7 SDS-PAGE and Western Blot analysis

SDS-PAGE (sodium dodecyl polyacrylamide gel electrophoresis) was performed under reducing conditions with a 10.8% polyacrylamide resolving gel and

a 3.9% polyacrylamide stacking gel in a vertical electrophoresis chamber (Penguin™ Water-Cooled Dual-Gel Electrophoresis System, Peqlab, Erlangen, Germany). For semidry blotting of the proteins on nitrocellulose membrane (Immobilon-NC HAHY, Sigma-Aldrich) a 2117 Multiphor II transfer unit (Pharmacia, Freiburg, Germany) was used.

After blocking non-specific protein binding with 1% fish gelatine in TRIS buffered saline (TBS, pH 7.4), the membranes were incubated for two hours with the respective primary antibody. For β-actin, mouse anti-β-actin-IgG (clone AC-15, Sigma-Aldrich) was used at a dilution of 1:5000 and for COX-2 rabbit anti-COX-2-IgG (affinity purified, Biotrend, Cologne, Germany) was used at a dilution of 1:500. After washing twice with 0.05% Tween 20 in TBS, the membranes were incubated for two hours with the respective secondary antibody, alkaline phosphatase-conjugated sheep anti-mouse-IgG (Chemicon International, Hofheim, Germany) and alkaline phosphatase-conjugated goat anti-rabbit-IgG (Rockland, Gilbertsville, PA, USA) at a dilution of 1:5000. Colorimetric detection was performed with Sigma Fast™ BCIP/NBT substrate (Sigma-Aldrich), according to the manufacturer's protocol.

2.8 Statistical analysis

To compensate the variation between single experiments, measurements were adjusted to accompanying zero control samples. Therefore all data are expressed as the percentage increase of PGE_2 concentration compared to zero control.

For statistical calculation of differences between the PGE_2 concentration in the same treatment groups of control and drug-perfused uteri, the Mann-Whitney rank sum test for unpaired matches (U test) was performed on the adjusted data.

3 Results

3.1 Prostaglandin E_2 concentration in myometrial tissue

The measured PGE_2 concentrations in zero control biopsies ranged between 0.1 and 2.2 ng/mg protein. While hemoper-

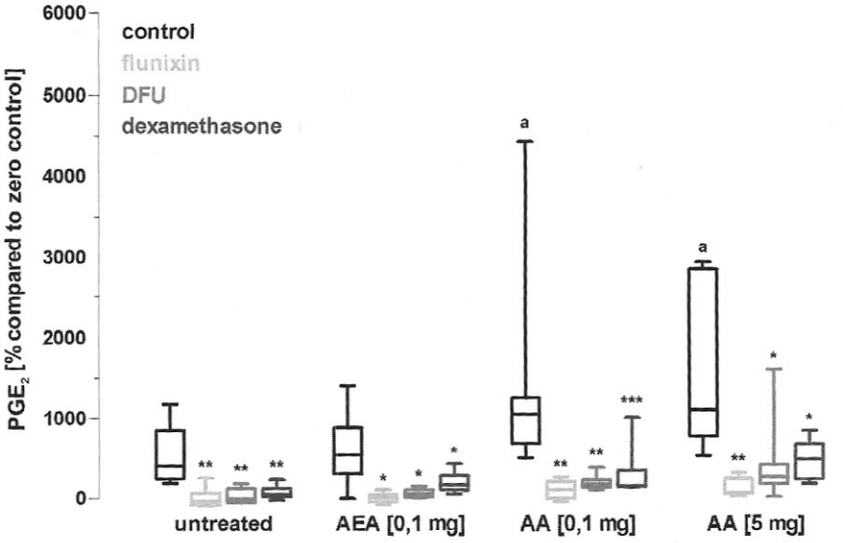

Fig. 3: PGE_2 concentration (percent of zero control, zero control = 0%) in uterine tissue after three hours of hemoperfusion and stimulation with AA (0.1 or 5 mg) and AEA (0.1 mg). Significant inhibition of PGE_2 synthesis in all treatment groups by perfusion with flunixin (1 µg/ml, n = 5), DFU (0.36 µg/ml, n = 6) and dexamethasone (0.1 µg/ml, n = 6), compared to control uteri (n = 6). *, $p < 0.05$; **, $p < 0.01$; ***, $p \leq 0.001$ (U test); a: $p < 0.05$ (U test) compared to untreated control.

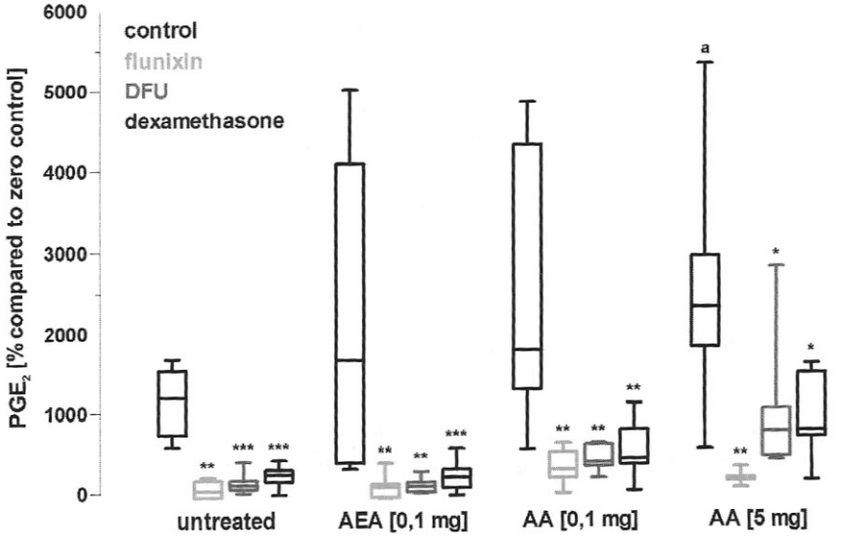

Fig. 4: PGE_2 concentration (percent of zero control, zero control = 0%) in uterine tissue after five hours of hemoperfusion and stimulation with AA (0.1 and 5 mg) and AEA (0.1 mg). Significant inhibition of PGE_2 synthesis in all treatment groups by perfusion with flunixin (1 µg/ml, n = 5), DFU (0.36 µg/ml, n = 6) and dexamethasone (0.1 µg/ml, n = 6), compared to control uteri (n = 6). *, $p < 0.05$; **, $p < 0.01$; ***, $p \leq 0.001$ (U test); a: $p < 0.05$ (U test) compared to untreated control.

fusion itself induced a distinct increase in the PGE_2 concentration in untreated myometrial tissue, AA (5 and 0.1 mg) treated tissue contained a significantly ($p < 0.05$) higher concentration of PGE_2 compared to untreated tissue after 180 minutes of hemoperfusion (Fig. 3). After 300 minutes of hemoperfusion PGE_2 concentration was increased even more prominently, though a significant difference was only observed between the 5 mg AA-treated and the untreated tissue (Fig. 4). Treatment with AEA resulted only in a tendency towards increased tissue PGE_2 that was not statistically significant after both 180 and 300 minutes of hemoperfusion (Fig. 3, 4).

Compared to hemoperfused control uteri, perfusion with anti-inflammatory test compounds resulted in a significant reduction of the PGE_2 concentration in the uterine tissue in all treatment groups at 180 and 300 minutes (Fig. 3, 4). Comparison between the test compounds revealed a significantly lower PGE_2 concentration in 5 mg AA treated tissue of flunixin-perfused uteri compared to dexamethasone perfusion at 180 ($p < 0.01$) and 300 minutes ($p < 0.05$) and to DFU perfusion at 300 minutes ($p < 0.01$).

3.2 Regulation of COX-1, COX-2 and iNOS

RT-PCR analysis of COX-1 mRNA showed no changes in the intensity of the bands at 0, 180 and 300 minutes of hemoperfusion, while there was a clear increase in the intensity of the mRNA bands of COX-2 and iNOS after three and five hours of hemoperfusion (Fig. 5). On the protein level, COX-2 was already present in the myometrium before reperfusion and there was no change in the band intensity observed over the perfusion time (Fig. 6).

In uteri perfused with dexamethasone, the intensity of the COX-1 mRNA bands also remains unchanged, while the intensity of the COX-2 mRNA bands increased distinctly after 180 and 300 minutes of hemoperfusion (Fig. 5). As observed in control uteri, COX-2 protein was already present in the myometrium before reperfusion and did not change over the perfusion time (Fig. 6). The band intensity of iNOS mRNA was enhanced after 180 minutes and de-

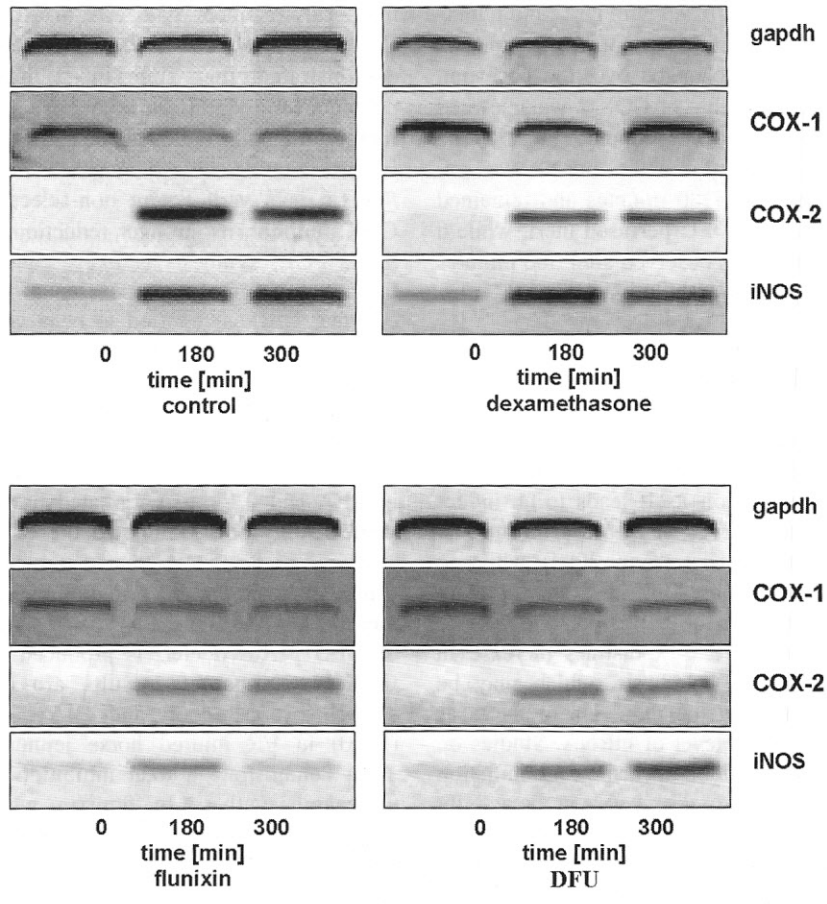

Fig. 5: Representative results of RT-PCR analysis (out of four) of tissue biopsies taken from isolated hemoperfused bovine uteri over a perfusion time of 300 minutes. Band intensity of COX-1, COX-2 and iNOS mRNA adjusted to GAPDH, influence of perfusion with antiinflammatory test substances.

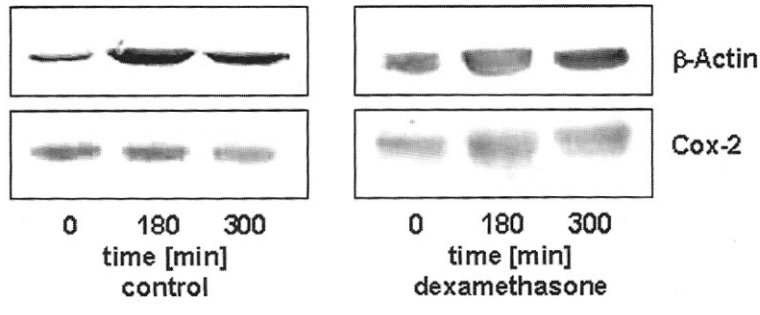

Fig. 6: Representative results of Western Blot analysis (out of four) of tissue biopsies taken from isolated hemoperfused bovine uteri over a perfusion time of 300 minutes. Band intensity of COX-2 protein adjusted to β-actin, influence of perfusion with dexamethasone.

creased slightly after 300 minutes of perfusion (Fig. 5).

In uteri perfused with DFU and flunixin, the band intensity of COX-1 mRNA was also unchanged over the perfusion time, while that of COX-2 was elevated after 180 and 300 minutes (Fig. 5). The band intensity of iNOS mRNA was enhanced after 180 minutes and remained elevated in DFU perfused uteri, while a decrease was observed after 300 minutes of perfusion with flunixin (Fig. 5).

4 Discussion

As previously demonstrated, after a period of ischemia in the isolated uterus, hemoperfusion itself leads to an induction of COX-2 on the level of mRNA (Braun and Kietzmann, 2004), while no change is observed in COX-2 protein amount over the perfusion time. Nevertheless, there is a distinct increase in tissue PGE_2 synthesis, which may be explained by the induction of iNOS as seen on the level of mRNA. Studies on animal models of acute inflammation describe a crosslink between the activity of COX-2 and iNOS. Reduced PGE_2 production in inflamed tissue is observed after inhibition of the NO producing enzyme (Salvemini et al., 1993; Salvemini et al., 1995). Further evidence for the involvement of NO in the catalytic activity of COX-2 was given by Landino et al. (1996), who reported that peroxynitrite (the product of NO and the superoxide radical) is able to activate the COX even in the presence of potent endogenous radical scavengers, such as glutathione and glutathione peroxidase. So, the induction of iNOS, as well as an enhanced production of peroxynitrite during reperfusion are typical events which occur during I/R-injury (Hur et al., 1999; Carden and Granger, 2000; Chan, 2002). Therefore, the enhanced PGE_2 synthesis in the (re-)perfused uterine tissue may be explained by an activation of COX-2 by peroxynitrite, while the perfusion time of five to six hours is not sufficiently long to result in a measurable increase of COX-2 protein synthesis.

Perfusion of the organs with the test substances dexamethasone, flunixin and DFU resulted in a significant inhibition of tissue PGE_2 synthesis, regardless of the local treatment with AA or AEA. Comparison of the tested substances demonstrated that flunixin is most potent, with a significantly higher COX inhibition than dexamethasone and DFU in tissue stimulated with 5 mg AA. Since flunixin is a very potent non-selective COX inhibitor, its stronger reduction of tissue PGE_2 synthesis is likely caused by the inhibition of both isoforms of the enzyme. DFU is reported to be a very selective COX-2 inhibitor with a 1000 times higher affinity to COX-2 than to COX-1 (Riendeau et al., 1997). Therefore DFU is able to inhibit the preferentially COX-2-mediated PGE_2 synthesis in AEA and 0.1 mg AA treated tissue, while it only partly inhibited the PGE_2 synthesis in 5 mg AA treated tissue. It can be assumed that the remaining PGE_2 measured in the 5 mg AA treated tissue of DFU perfused uteri is produced by COX-1. Comparable results are described by Campbell and Blikslager (2000) in I/R injured horse jejunum. They demonstrate a total inhibition of eicosanoid synthesis by flunixin, while a COX-2 selective inhibitor (etodolac) inhibits the PGE_2 synthesis to a lesser extent. As expected, neither flunixin nor DFU influence the regulation of COX-2 mRNA. Interestingly, there seemed to be a down-regulatory effect of flunixin on the iNOS mRNA after 300 minutes of perfusion. To our knowledge, comparable data for flunixin are not available in literature, whereas Mariotto et al. (1995) describe a significant reduction of iNOS mRNA in the lipopolysaccharide-stimulated rat stomach by treatment with the NSAIDs flurbiprofene and nitroflurbiprofene.

Dexamethasone on the other hand acts via different mechanisms, implying the inhibition of the phospholipase (PL) A_2 and the down-regulation of proinflammatory enzyme and cytokine expression on the level of mRNA and protein (Ruzicka, 1984; Masferrer et al., 1992; Salvemini et al., 1995; Michel et al., 1995; Whelan et al., 1999). Since PLA_2 liberates the COX substrate AA from membrane phospholipids, its function is overridden by treatment with AA and AEA in the present experimental design. Therefore it is unlikely that dexamethasone exhibits its effect on PGE_2 synthesis in the hemoperfused myometrium via inhibition of PLA_2. Regarding mRNA and protein expression in dexamethasone-perfused uteri there is also no influence on the regulation of the COX-2 observed, though it is a commonly reported mechanism by which glucocorticoids modulate the inflammatory response in animal models of acute inflammation (Masferrer et al., 1990; Utsunomiya et al., 1994; Harada et al., 1996; Zhang et al., 1997; Kawamura et al., 2000). The missing effect is likely explained by the time of application, which starts with the onset of hemoperfusion. As the trigger of the inflammatory reaction coincides with the beginning of dexamethasone application, the perfusion time may not be sufficient to develop the inhibitory effect. Therefore, to achieve an observable inhibition of COX-2 expression, the administration of dexamethasone before reperfusion would be necessary. However, since this would mean a systemic treatment of the animals right before slaughter, i.e. an animal experiment, it is impossible to implement such a preceding administration in the described *in vitro* study. A prolonged perfusion time should also be avoided, since sufficient organ viability cannot be ensured over a longer time period.

Since an inhibitory effect of dexamethasone on COX-2 expression can be excluded, the mode of action by which it reduces PGE_2 synthesis in the uterine tissue needs to be elucidated. A direct inhibition of enzyme activity cannot be excluded, though it is unlikely, since no similar findings are reported in the literature. Although it was not examined in this study, it is more likely that dexamethasone reduces eicosanoid synthesis by inhibiting the expression of subsequent enzymes, like the PGE synthase, as reported by Stichtenoth et al. (2001) in primary rheumatoid synovial cells.

In conclusion, the isolated hemoperfused bovine uterus is a suitable *in vitro* model for the investigation of anti-inflammatory substances, including their COX selectivity. Regarding the prostaglandin synthesis the results of the present study

are comparable with those observed in common animal models of acute inflammation, like the tissue cage model in cattle, horses and dogs, the carrageenin induced pleurisy in rats and the AA induced ear swelling in mice (Espinasse et al., 1994; Landoni et al., 1995; Harada et al., 1996; Puigneró and Queralt, 1997). Since these models are associated with pain and stress, the provision of an *in vitro* model as an alternative approach is likely to reduce the suffering of the animals. Therefore, though it will not be a model of first choice in drug research, the isolated hemoperfused bovine uterus may contribute to both the reduction of animal testing and a further understanding of I/R-injury and inflammatory reactions, especially considering that there are variations between different models of acute inflammation concerning the COX selectivity of tested substances (Engelhardt et al., 1996a; 1996b).

References

Bäumer, W. and Kietzmann, M. (2000). The isolated perfused bovine udder as a model of dermal eicosanoid release. *Altern. Lab. Anim. 28*, 643-649.

Bäumer, W., Mertens, A., Braun, M. and Kietzmann, M. (2002). The isolated perfused bovine uterus as a model for mucous membrane irritation and inflammation. *ALTEX 19*, 57-63.

Braun, M. (2002). Der isoliert hämopefundierte Rinderuterus als In-vitro-Modell zur Prüfung antiinflammatorischer Substanzen. Hannover, Tierärztliche Hochschule, *Thesis.*

Braun, M. and Kietzmann, M. (2004). Ischemia reperfusion injury in the isolated hemoperfused bovine uterus – an in vitro model of acute inflammation. *Altern. Lab. Anim.*, accepted for publication.

Campbell, N. B. and Blikslager, A. T. (2000). The role of cyclooxygenase inhibitors in repair of ischaemic-injured jejunal mucosa in the horse. *Equine. Vet. J. Suppl.,* 59-64.

Carden, D. L. and Granger, D. N. (2000). Pathophysiology of ischaemia-reperfusion injury. *J. Pathol. 190*, 255-266.

Chan, K. L. (2002). Role of nitric oxide in ischemia and reperfusion injury. *Curr. Med. Chem. – Anti-Inflammatory & Anti-Allergy Agents 1*, 1-13.

Engelhardt, G., Bögel, R., Schnitzer, C. and Utzmann, R. (1996a). Meloxicam: influence on arachidonic acid metabolism. Part I. In vitro findings. *Biochem. Pharmacol. 51*, 21-28.

Engelhardt, G., Bögel, R., Schnitzer, C. and Utzmann, R. (1996b). Meloxicam: influence on arachidonic acid metabolism. Part II. In vivo findings. *Biochem. Pharmacol. 51*, 29-38.

Espinasse, J., Thouvenot, J. P., Dalle, S. et al. (1994). Comparative study of the action of flunixin meglumine and tolfenamic acid on prostaglandin E_2 synthesis in bovine inflammatory exudate. *J. Vet. Pharmacol. Ther. 17*, 271-274.

Fu, J. Y., Masferrer, J. L., Seibert, K. et al. (1990). The induction and suppression of prostaglandin H_2 synthase (cyclooxygenase) in human monocytes. *J. Biol. Chem. 265*, 16737-16740.

Hamberg, M. and Samuelsson, B. (1967). On the mechanism of the biosynthesis of prostaglandins E_1 and $F_{1\alpha}$. *J. Biol. Chem. 242*, 5336-5343.

Harada, Y., Hatanaka, K., Kawamura, M. et al. (1996). Role of prostaglandin H synthase-2 in prostaglandin E_2 formation in rat carrageenin-induced pleurisy. *Prostaglandins 51*, 19-33.

Herschman, H. R. (1996). Prostaglandin synthase 2. *Biochim. Biophys. Acta 1299*, 125-140.

Hinz, B. and Brune, K. (2002). Cyclooxygenase-2 – 10 years later. *J. Pharmacol. Exp. Ther. 300*, 367-375.

Hur, G. M., Ryu, Y. S., Yun, H. Y. et al. (1999). Hepatic ischemia/reperfusion in rats induces iNOS gene transcription by activation of NF- κB. *Biochem. Biophys. Res. Commun. 261*, 917-922.

Kawamura, M., Hatanaka, K., Saito, M. et al. (2000). Are the anti-inflammatory effects of dexamethasone responsible for inhibition of the induction of enzymes involved in prostanoid formation in rat carrageenin-induced pleurisy? *Eur. J. Pharmacol. 400*, 127-135.

Kozak, K. R., Crews, B. C., Ray, J. L. et al. (2001). Metabolism of prostaglandin glycerol esters and prostaglandin ethanolamides in vitro and in vivo. *J. Biol. Chem. 276*, 36993-36998.

Landino, L. M., Crews, B. C., Timmons, M. D. et al. (1996). Peroxynitrite, the coupling product of nitric oxide and superoxide, activates prostaglandin biosynthesis. *Proc. Natl. Acad. Sci. U. S. A. 93*, 15069-15074.

Landoni, M. F., Cunningham, F. M. and Lees, P. (1995). Determination of pharmacokinetics and pharmacodynamics of flunixin in calves by use of pharmacokinestic/pharmacodynamic modeling. *Am. J. Vet. Res. 56*, 786-794.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem. 193*, 265-275.

Mariotto, S., Menegazzi, M., Carcereri de Prati, A. et al. (1995). Protective effect of NO on gastric lesions and inhibition of expression of gastric inducible NOS by flurbiprofen and its nitro-derivative, nitroflurbiprofen. *Br. J. Pharmacol. 116*, 1713-1714.

Masferrer, J. L., Zweifel, B. S., Seibert, K. and Needleman, P. (1990). Selective regulation of cellular cyclooxygenase by dexamethasone and endotoxin in mice. *J. Clin. Invest. 86*, 1375-1379.

Masferrer, J. L., Seibert, K., Zweifel, B. and Needleman, P. (1992). Endogenous glucocorticoids regulate an inducible cyclooxygenase enzyme. *Proc. Natl. Acad. Sci. U. S. A. 89*, 3917-3921.

Mertens, A. (2001). Untersuchungen zur Schleimhautverträglichkeit von Testsubstanzen am Modell des isoliert perfundierten Rinderuterus. Hannover, Tierärztliche Hochschule, *Thesis.*

Michel, G., Nowok, K., Beetz, A. et al. (1995). Novel steroid derivative modulates gene expression of cytokines and growth regulators. *Skin Pharmacol. 8*, 215-220.

Puigneró, V. and Queralt, J. (1997). Effect of topically applied cyclooxygenase-2-selective inhibitors on arachidonic acid- and tetradecanoylphorbol acetate-induced dermal inflammation in the mouse. *Inflammation 21*, 431-442.

Riendeau, D., Percival, M. D., Boyce, S. et al. (1997). Biochemical and pharmacological profile of a tetrasubstituted furanone as a highly selective COX-2 inhibitor. *Br. J. Pharmacol. 121*, 105-117.

Ruzicka, T. (1984). Stoffwechsel der Arachidonsäure in der Haut und seine Bedeutung in der Pathophysiologie entzündlicher Dermatosen. *Hautarzt 35*, 337-343.

Salvemini, D., Misko, T. P., Masferrer, J. L. et al. (1993). Nitric oxide activates cyclooxygenase enzymes. *Proc. Natl. Acad. Sci. U. S. A. 90*, 7240-7244.

Salvemini, D., Settle, S. L., Masferrer, J. L. et al. (1995). Regulation of prostaglandin production by nitric oxide; an in vivo analysis. *Br. J. Pharmacol. 114*, 1171-1178.

Shitashige, M., Morita, I. and Murota, S. (1998). Different substrate utilization between prostaglandin endoperoxide H synthase-1 and -2 in NIH3T3 fibroblasts. *Biochim. Biophys. Acta 1389*, 57-66.

Stichtenoth, D. O., Thorén, S., Bian, H. et al. (2001). Microsomal prostaglandin E synthase is regulated by proinflammatory cytokines and glucocorticoids in primary rheumatoid synovial cells. *J. Immunol. 167*, 469-474.

Utsunomiya, I., Nagai, S. and Oh-ishi, S. (1994). Differential effects of indomethacin and dexamethasone on cytokine production in carrageenin-induced rat pleurisy. *Eur. J. Pharmacol. 252*, 213-218.

Whelan, C. J., Payne, A. N. and Planquois, J. M. (1999). A comparison of the inhibitory effects of budesonide, beclomethasone dipropionate, dexamethasone, hydrocortisone and tixocortol pivalate on cytokine release from leukocytes recovered from human bronchoalveolar lavage. *Inflamm. Res. 48*, 224-228.

Xie, W. L., Chipman, J. G., Robertson, D. L. et al. (1991). Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. *Proc. Natl. Acad. Sci. U. S. A. 88*, 2692-2696.

Yu, M., Ives, D. and Ramesha, C. S. (1997). Synthesis of prostaglandin E_2 ethanolamide from anandamide by cyclooxygenase-2. *J. Biol. Chem. 272*, 21181-21186.

Zhang, Y., Shaffer, A., Portanova, J. et al. (1997). Inhibition of cyclooxygenase-2 rapidly reverses inflammatory hyperalgesia and prostaglandin E_2 production. *J. Pharmacol. Exp. Ther. 283*, 1069-1075.

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