M3 receptor as a key target of N,N-diethyl-m-toluamide (DEET) to promote angiogenesis

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Oral Communication Abstracts

PHARMACOLOGIE CARDIOVASCULAIRE

CO-001

Cutaneous iontophoresis of treprostinil, a prostacyclin analogue, increases microvascular blood flux in diabetic malleolus area

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Introduction: Diabetic foot ulcers are one of the most common and serious complications of diabetes mellitus. Few drugs are effective into enhancing the healing of microvascular skin ulcers. The main objective of the present study was to determine whether iontophoresis of treprostinil, a prostacyclin analogue, increases skin microvascular blood flux in the malleolus area of healthy subjects and diabetic patients.

Material and methods: We recruited 12 healthy subjects and 12 type 2 diabetic patients. Cathodal iontophoresis (40 mC/cm²) of treprostinil 250 μM and NaCl 0.9% was performed in the malleolus area. Skin hyperaemia was quantified using non-invasive laser speckle contrast imaging, and expressed as cutaneous vascular conductance (CVC).

Results: In healthy controls and diabetic patients, treprostinil 250 μM induced a significant increase in CVC compared with NaCl (for diabetic patients, AUC0-4 h was 16181 + /- 6709; vs. 2219 + /- 3616% BL.min, respectively; $P = 0.002$). In both groups, peak hyperaemia was obtained between 30 min and 1 h after the end of treprostinil iontophoresis and flux remained higher than baseline up to 6 h after the end of iontophoresis. No significant side-effect occurred.

Discussion/Conclusion: Cutaneous iontophoresis of 250 μM treprostinil increases microvascular blood flux in malleolus area in healthy volunteers and diabetic patients, without inducing systemic or local side-effects. Topical administration of prostacyclin analogue administered through iontophoresis combines the advantages of drug therapy and local low-intensity current application providing a potential innovative, well-tolerated therapy of microvascular skin ulcers. Treprostinil cathodal iontophoresis should be further investigated as a new local therapy for diabetic ulcers.

CO-002

Regular exercise activates cardioprotective signalling pathways but does not induce mitochondrial adaptations in both lean and obese mice in the absence of myocardial ischemia

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Introduction: Comorbidities such as obesity are serious risk factors for cardiovascular diseases and prevent the ability of cardioprotective strategies to protect the myocardium against infarction. We particularly reported that post-conditioning failed to reduce infarct size in obese mice secondary to dysregulation of Akt and ERK1/2 pathways. However, we recently showed that regular exercise was able to restore cardioprotection in obese mice by increasing the phosphorylation state of these kinases and consequently by improving the resistance of mitochondrial permeability transition pore (mPTP) to opening at the onset of coronary artery reperfusion. This study aimed at determine whether the beneficial adaptations induced by exercise develop as a pre-emptive mechanism, i.e., before ischemia-reperfusion or rather specifically at reperfusion.

Material and methods: Wild-type (WT) and obese (ob/ob) mice were assigned to sedentary conditions or regular treadmill exercise (1 h/day, 5 days/7, 4 weeks, 4° slope, 10–30 cm/s). Pro-survival kinases involved in the Reperfusion Injury Salvage Kinase pathway, as Akt, ERK1/2 and PKCε and the corresponding phosphatases, PTEN and MKP-3, were studied using Western Blot analysis in the absence of ischemic event. We also investigated mitochondrial respiration and mPTP opening was assessed by monitoring mitochondrial calcium retention capacity.

Results: Exercise significantly increased the phosphorylation of Akt and ERK1/2 and concomitantly reduced the expression of the corresponding phosphatases PTEN and MKP-3 in both WT and ob/ob animals. Moreover, it induced a translocation of PKCε from the cytosol to mitochondria in WT and in ob/ob mice. Importantly, despite the modifications induced in the phosphorylation state of these kinases, exercise did not affect the calcium concentration required to open mPTP and respiratory parameters neither in WT nor in ob/ob animals.

Discussion/Conclusion: Although exercise enhanced the phosphorylation of the pro-survival kinases, it did not induce pre-emptive mitochondrial adaptations that are known to be mandatory for cardioprotection. Therefore, this activation of survival pathways needs to be combined with stress conditions, i.e., ischemia-reperfusion, to initiate downstream signalling pathways which converge to mitochondria and inhibit mPTP opening at the onset of reperfusion.

CO-003

Chronic oral intake of the omega 3 EPA:DHA 6:1 formulation protects against Angiotensin II-induced hypertension and endothelial dysfunction in rats

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Introduction: Regular intake of fish products is associated with a reduced risk of cardiovascular diseases. The beneficial effect has been attributed at least in part to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). EPA and DHA have been shown to cause endothelium-dependent nitric oxide (NO)-mediated relaxations of isolated blood vessels, with an optimized ratio of EPA:DHA 6:1. The aim of the present study was to determine whether chronic intake of EPA:DHA 6:1 affects hypertension and endothelial dysfunction induced by angiotensin II (Ang II).

Material and methods: Male Wistar rats daily received 500 mg/kg of either EPA:DHA 6:1 (omega 3) or corn oil (control) for 5 weeks. After 1 week, rats underwent sham surgery or surgery with implantation of an osmotic mini-pump infusing Ang II (0.4 mg/kg/days) for 4 weeks. Blood pressure was monitored by tail sphyngomanometry, and the reactivity of second branch mesenteric artery rings using myographs.

Results: Infusion of Ang II to rats induced within 7 days a pronounced increase of systolic blood pressure, which reached 215.6 ± 8.5 mmHg compared to 136.8 ± 6.2 mmHg ($n = 8$) in the control group after 21 days. The hypertensive response to Ang II was markedly reduced in the omega 3 group reaching 169.0 ± 7.8 mmHg whereas the omega 3 treatment alone had no effect (136.0 ± 4.2 mmHg). In second branch mesenteric arteries, relaxations to acetylcholine (Ach) were markedly reduced in the Ang II group affecting the endothelium-dependent hyperpolarization (EDH)-mediated component to a greater extent than the NO-mediated component. The NAPDH oxidase inhibitor (VAS-2870) improved both components in the Ang II group. Pronounced endothelium-dependent contractile responses to Ach were observed in the Ang II group compared to the control group, which were abolished by indomethacin. Chronic intake of EPA:DHA 6:1 prevented the Ang II-induced endothelial dysfunction both by improving the relaxations and by reducing endothelium-dependent contractile responses to Ach.

Discussion/Conclusion: The present findings indicate that chronic intake of EPA:DHA 6:1 prevented the development of hypertension and endothelial dysfunction induced by the infusion of Ang II to rats. The Ang II-induced endothelial dysfunction involves NADPH oxidase, indicating a redox-sensitive mechanism. The beneficial effect of EPA:DHA 6:1 is mediated by an improvement of both the NO- and the EDH-mediated relaxations as well as a reduction of endothelium-dependent contractile response most likely by preventing oxidative stress.

PHARMACOLOGIE MOLÉCULAIRE ET PRÉ-CLINIQUE

CO-004

Chronic oral intake of a standardized Crataegus extract prevents DOCA-salt-induced hypertension, and alteration of cardiac, vascular and renal structures and functions in rats: Role of oxidative stress

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Introduction: Hypertension is a leading risk factor for the development and progression of chronic heart, vascular and renal diseases. The hypertension-induced end target organ damage is characterized by alterations of the heart, vascular and kidney functions and structure associated with oxidative stress. The present study has examined the possibility that a standardized polyphenol-rich Crataegus extract prevents hypertension-induced end target organ damage in an experimental model of hypertension, the DOCA-salt non-nephrectomized rat.

Material and methods: Male Wistar rats were divided into a control group, a Crataegus extract group (100 or 300 mg/kg/day in the diet), a DOCA-salt group (50 mg/kg i.p. per week) and DOCA-salt + Crataegus extract group for 6 weeks. Systolic arterial blood pressure (SBP) and target organ damage (vasculature, heart and kidney) were examined.

Results: The DOCA-salt treatment increased systolic blood pressure, and this effect was associated with the induction of pronounced endothelium-dependent contractile responses in second order mesenteric resistance artery rings to acetylcholine. The increased contractile response was prevented by indomethacin (a cyclooxygenase inhibitor) and associated with vascular oxidative stress as assessed using dihydroethidine, a reduced expression of eNOS, and an increased expression of NADPH oxidase subunits (gp91 phox, p47 phox), COX-1 and COX-2. Echocardiography and histological analyses indicated left ventricular (LV) hypertrophy, fibrosis, and impaired systolic and diastolic cardiac functions associated with oxidative stress in the DOCA-salt group. Renal dysfunction characterized by increased urea and uric acid plasma levels, glomerular and arteriolar hypertrophy, tubulo-interstitial fibrosis associated with oxidative stress was observed in the DOCA-salt group.

Discussion/Conclusion: Chronic intake of the Crataegus extract significantly reduced in a dose-dependent manner systolic blood pressure, target end organ damage in the heart and kidney, and improved the vascular function by preventing oxidative stress and the expression of target molecules. Thus, the Crataegus extract was able to retard the hypertension-induced end organ damage in the heart, kidney and resistance arteries most likely by preventing oxidative stress.

CO-005

M3 receptor as a key target of N,N-diethyl-m-toluamide (DEET) to promote angiogenesis

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Introduction: It has been suggested that muscarinic pathway can lead to pro-angiogenic effects in endothelial cells. The insect repellent N,N-diethyl-m-toluamide (DEET) recently proposed as an inhibitor of human acetylcholinesterase has been reported to potentiate tumor development. As angiogenesis is a criti-

cal step in tumorigenesis and since it has been demonstrated that donepezil, another inhibitor of human acetylcholinesterase, is able to promote angiogenesis, the present study was designed to test the potential effect of DEET on different processes leading to neovascularization.
Material and methods: Human Umbilical Vein Endothelial Cells (HUVECs) were incubated for 24 h with DEET at concentrations similar to that found in plasma of exposed individuals (10^{-5}M) or in the environment (10^{-8}M). Calcium signaling in presence of both concentrations of DEET was also investigated in HUVEC and in human embryonic kidney (HEK) 293 cells expressing recombinant Galpha (q/11)-coupled muscarinic M3 receptors (HEK/M3). *In vivo* vascularization was assessed by using Matrigel® plug model and U87MG tumor cells xenagraft in *Nude* mice daily treated with DEET for 28 days. Involvement of muscarinic pathway was evaluated with selective muscarinic M3 antagonist parafluorohexahydrosiladiphenidol (pFHHSiD) or with M3 siRNA.
Results: Both concentrations of DEET increased proliferation, migration and adhesion, in vitro capillary length of HUVECS in culture on matrigel and in vivo vascularization of Matrigel® Plug. These cellular processes were associated with focal adhesion kinase (FAK) phosphorylation and stress fibers. Both concentrations of DEET also stimulated NO production through an increase of peNOS-Ser/peNOS-Thr ratio. Blockade or silencing M3 muscarinic receptor subtype abolished the pro-angiogenic properties of DEET in both *in vitro* and *in vivo* conditions. DEET also potentiates carbachol-induced calcium signaling in HEK/M3. Moreover, tumorigenesis following U87MG cells injection in Nude mice was increased by administration of DEET due to enhanced tumor microvascular density.
Discussion/Conclusion: These data underscore a novel property of DEET as a pro-angiogenic toxic, *via* the involvement of M3 muscarinic receptor subtype that could explain its potentiating effect on tumor development [1-3].
References:
[1] Kakinuma Y. et al., Donepezil, an acetylcholinesterase inhibitor against Alzheimer's dementia, promotes angiogenesis in an ischemic hindlimb model. J. Mol. Cell. Cardiol. (2010) 48, p. 680–693.
[2] Fradin M.S., Day J.F. Comparative efficacy of insect repellents against mosquito bites. New Eng. J. Med. (2002) 4, p. 3–8.
[3] Corbel V. et al., Evidence for inhibition of acetylcholinesterases in insect and mammalian nervous systems by the insect repellent deet. BMC Biology, (2009) 5, 7:47, p. 1–11.

CO-006
Etanercept improves endothelial function in Rat Adjuvant-Induced Arthritis: mechanisms involved
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Introduction: Growing evidence indicate that increased cardiovascular risk associated to Rheumatoid Arthritis (RA) is secondary to the presence of endothelial dysfunction (ED) [1]. To date, the effect of anti-arthritic drugs used on RA-induced ED is still controversial [2-3]. In the present study, we investigated the effect of the anti-TNFα Etanercept on endothelial function in the model of adjuvant-induced arthritis (AIA) rats.
Material and methods: AIA was induced by an intradermal injection of Mycobacterium butyricum in the tail of male Lewis rats. At the first signs of arthritis, AIA rats received Etanercept (10 mg/kg/ 3 days, s.c) or saline (control AIA) for 21 days. Arthritis score was daily evaluated. At the end of treatment, endothelial function was studied in pre-constricted aortic rings relaxed by cumulative concentrations of acetylcholine (Ach, 10^{-11}-10^{-4} moles/liter) in the presence or not of inhibitors of NOS (L-NAME), COX-2 (NS398), Arginase (nor-NOHA), EDHF (apamin/charybdotoxin) and superoxide anions production (Tempol). Aortic expression of eNOS, phospho-eNOS, COX-2, Arginase-2, p22phox and p47phox was evaluated by western blotting.
Results: As compared to control AIA, Etanercept significantly reduced arthritis score (−34%, $P < 0.001$). This effect was associated with a significant improvement of Ach-induced relaxation ($P < 0.05$). However, no correlation was found between the arthritis score and Ach-induced relaxation (Emax) ($r = -0.195$; $P = 0.246$, all AIA rats). The results showed that the beneficial effect of Etanercept relied on increase in NOS activity and a decrease in COX-2 and Arginase activities as well as $O_2^{\cdot-}$ production. These effects are secondary to increased eNOS expression and phosphorylation and decreased COX-2, Arginase-2 and p22phox expressions. By contrast, EDHF production and p47 expression were unaffected by the treatment.
Discussion/Conclusion: Our data demonstrated that Etanercept improved endothelial function in AIA, via an impact on the NOS, Arginase, COX-2 and NADPH oxidase pathways. The lack of correlation between endothelial function and arthritic score in AIA suggests that improved endothelial function evoked by Etanercept is independent on the reduction of systemic inflammation but rather related to direct vascular effects.
References:
[1] Sandoo A. et al. Vascular function and morphology in rheumatoid arthritis: a systematic review. Rheumatology (Oxford) 2011;50:2125–2139.
[2] van Breukelen-van der Stoep D.F. et al. Cardiovascular risk in rheumatoid arthritis: how to lower the risk? Atherosclerosis. 2013 Nov;231(1):163–72.
[3] Steyers CM 3rd, Miller FJ Jr. Endothelial dysfunction in chronic inflammatory diseases. Int J Mol Sci. 2014 Jun;15(7):11324–49.

CO-007
Imidazoline-like drug improve insulin sensitivity through a direct stimulation of adiponectin secretion in primary adipocytes
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Introduction: Altered adiponectin (a major insulin sensitizer adipokine) signaling has been proposed as a key factor in the development of insulin resistance and thus, in the pathogenesis of metabolic syndrome. We previously showed that imidazoline I_1 receptor (I_1R) ligands can improve insulin sensitivity and glucose tolerance partly through increased adiponectin plasma levels. The objective of this study was to explore possible direct actions of I_1R ligands on adipocytes.
Material and methods: Experiments were carried out in primary adipocytes isolated from the epididymal white adipose tissue of aged male Wistar rats. Expression of I_1R on adipocytes was verified with competition binding assays using [125I] para-iodoclonidine. In order to evaluate the effect of LNPs on adiponectin secretion, primary adipocytes were treated with increasing concentrations of LNP509, an I_1R ligand, in the presence or not of the I_1R antagonist efaroxan. In order to identify the intracellular signaling pathways involved in the action of LNPs, wortmannin (PI3K-inhibitor), IBMX (phosphodiesterase blocker) and AS1842856 (FOXO1 inhibitor) were added 45 min prior LNP509 exposure. After 3 h, the culture medium was collected and adiponectin content was measured by ELISA assay.
Results: Competition binding assays confirmed the expression of I_1R in adipocyte membrane. Treatment by LNP509 dose-dependently induced adiponectin secretion: from 18 ± 5 ng/mL at 10^{-9} M to 66 ± 15 ng/mL at 10^{-4} M; $P \leq 0.05$ vs. medium alone (25 ng/mL). Interestingly, the adiponectin secretion induced by the highest LNP509 concentration was similar to the one elicited by 10 µM insulin (61 ng/mL); the secretion was totally prevented by a pretreatment of the cells with efaroxan. The LNP509-induced adipokine secretion was unchanged by PI3K-inhibition, but was reduced after phosphodiesterase and FOXO1 blockade (−80% and −44% respectively, compared to LNP509 alone).
Discussion/Conclusion: Our data show that I_1R ligands directly act on adipocytes to promote adiponectin secretion. Since LNP-induced secretion of adiponectin was almost abolished by enhancing cAMP levels and was largely reduced after FOXO1 inhibition, the following transduction cascade can be proposed: I_1R → inactivation of adenylate cyclase → increase of cAMP → inactivation of SIRT1/FOXO1 → activation of PPARg → increase of adiponectin synthesis. Future studies should confirm the proposed signaling pathways.

CO-008
Study of the antiepileptic drugs transport through the blood-brain barrier in children
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Introduction: Drug resistant epilepsy remains a major concern in pediatrics despite the large number of anti-epileptic drug (AEDs) available. Resistance to AEDs may be related to adaptive mechanisms that oppose their brain penetration. Recent research has covered the role of the adult Blood-Brain Barrier (BBB) in this protective mechanism through altered expression of efflux pumps e.g. P-glycoprotein (P-gp) and Breast Cancer Resistance protein (BCRP). In the present work we aimed at studying the impact of valproic acid (VPA) on P-gp, BCRP efflux transporters function and in the expression of selected ABC and SLC transporters genes in immature rat brain microvessels.
Material and methods: Post-natal day 21 (P21) and P56 rats ($n = 5$) were treated for 5 days with VPA (400 mg/Kg/days and 350 mg/Kg/days) through subcutaneous osmotic pumps. Changes in BBB transporters P-gP and BCRP function was measured after the infusion of digoxin and prazosin as P-gP and BCRP substrates respectively and calculation of brain/blood partition coefficients (Kp, brain) in steady-state conditions. For gene expression analysis, rat brain microvessels were extracted and the mRNA levels were semi-quantified by qRT-PCR. Data were analyzed by 2-way ANOVA followed by Bonferoni-corrected t-student post-hoc analysis and considered significant if $P \leq 0.05$.
Results: After 5 days, digoxin Kp (mean±SD) increased from 0.070 ± 0.01 to 0.136 ± 0.016 in P21 rats and from 0.042 ± 0.02 to 0.077 ± 0.07 in P56, both differences being statistically significant ($P < 0.01$). A trend to decrease prazosin Kp (0.467 ± 0.057 vs. 0.370 ± 0.023) was noticed in P21 but was not statistically significant. Although P-gP and BCRP mRNA levels were significantly higher in P56 than in P21 rat brain microvessels (P-gP $P < 0.01$, BCRP $P < 0.05$), VPA treatment did not change both genes expression. Nonetheless, we observed a statistically significant 4-fold increase (2^{-DDCt}) in the expression of Slc22a6 gene (OAT1, organic anion transporter 1) after VPA treatment in P21 rats ($P < 0.01$), suggesting possible interaction between VPA and OAT1.
Discussion/Conclusion: P-gp function decreased significantly after VPA treatment in both P21 and P56 rats while a trend to increase BCRP function was observed only in P21 rats. VPA did not have an impact on the expression of P-gP and BCRP genes. OAT1 transporter expression was significantly increased in P21 rat brain microvessels after VPA treatment. Taken together, these results show a differential impact of VPA in efflux transporters at the blood brain barrier according to age.

CO-009
The promoting effect of phosphate and indoxyl sulfate on human aortic smooth muscle cells calcification is amplified by endothelial cells and may be associated to an inhibition of the osteopontin secretion
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Introduction: Vascular calcification is a consequence of processes involving the osteochondrogenic switch of vascular smooth muscle cells (SMCs) or their apoptosis. In chronic kidney disease (CKD), both intimal and medial calcifications are agravated. Indoxyl sulfate (IS), an indolic uremic toxin that accumulates in CKD patients, is known to increase endothelial cells (ECs) dysfunction and both IS and inorganic phosphate (Pi) accelerate vascular calcification. However, whether the endothelium is involved in promoting vascular calcification in CKD is not yet clarified.
Material and methods: We studied the role of ECs in the calcifications induced by human aortic SMCs exposed to Pi (3 mM) and IS (200 µM). In vitro calcifications were quantified by alizarin-red staining and viability of hASMCs and secre-

