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Role of ET_B and B_2 receptors in the *ex vivo* platelet inhibitory properties of endothelin and bradykinin in the mouse

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1 We have developed a model to study the inhibitory properties of endogenous autacoids triggered by systemically-administered vasoactive peptides, on platelet aggregation *ex vivo* in the mouse.

2 Adenosine diphosphate (ADP) (0.5–10 μM) induces a concentration-dependent aggregation of platelet-rich plasma derived from C57BL/6 mice. Intravenously-administered endothelin-1 (0.01–1 nmol kg^{-1}), the selective ET_B agonist, IRL-1620 (0.01–1 nmol kg^{-1}) or bradykinin (1–100 nmol kg^{-1}) significantly reduced in a dose-dependent fashion the ADP-induced platelet aggregation.

3 The non-selective cyclo-oxygenase (COX) inhibitor, indomethacin, a selective COX-2 inhibitor NS-398 or the prostacyclin synthase inhibitor, tranylcypromine (10 mg kg^{-1}), markedly reduced the inhibitory properties of endothelin-1, whereas only a combination of both indomethacin, NS-398 or tranylcypromine and L-NAME (10 mg kg^{-1}) were required to abolish the response to bradykinin.

4 An ET_B-selective antagonist (BQ-788) or knockout of the B_2 receptor gene (in B_2 knockout mice) abolishes the platelet inhibitory properties of endothelin-1 and bradykinin, respectively.

5 Our results suggest that intravenously-administered endothelin-1 and bradykinin, through ET_B and B_2 receptor activation, respectively, inhibit platelet aggregation *ex vivo* in the mouse. The inhibitory properties of endothelin-1 require the activation of COX-2 and the subsequent generation of prostacyclin. In addition to the two previously mentioned factors, nitric oxide is required for the anti-aggregatory effects of bradykinin.

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Abbreviations: ADP, adenosine diphosphate; B_2, B_2 bradykinin receptor; BQ-123, cyclo[D-Asp-L-Pro-D-Val-L-Leu-D-Trp]; BQ-788, (N,cis-2,6-dimethylpiperidinocarbonyl-L-γ-methylleucyl-D-1-methoxycarbonyl-tryptophanyl-D-norleucine); COX, cyclo-oxygenase; DMSO, dimethyl sulphoxide; ET_B, endothelin receptor (type B); $G\alpha_q$, subunit αq from G protein; Indo, indomethacin; IRL 1620, ET_B agonist; L-NAME, N^{ω}-nitro-L-arginine methyl ester; NO, nitric oxide; NOS, nitric oxide synthase; NS-398, COX-2 selective inhibitor; PBS, phosphate buffer saline; PGI_2, prostacyclin; PPP, platelet-poor plasma; PRP, platelet-rich plasma; TCP, tranylcypromine

Introduction

Several endogenous factors derived from the endothelium are involved in haemostatic mechanisms, such as platelet aggregation. Among other modulators, nitric oxide (NO) and eicosanoids influence the normal behaviour of blood platelets (Radomski *et al*., 1991; Herman *et al*., 1989a). It is also well established that intravenously (i.v.)-administered endothelin-1 (ET-1) triggers, *via* ET_B receptors, the release of an indomethacin-sensitive modulator of platelet aggregatory properties, as illustrated in several studies exploiting the *ex vivo* platelet aggregation method (McMurdo *et al*., 1993; Herman *et al*., 1989b).

The above-mentioned method is one of the few approaches to study the vasoactive peptide-induced release of endogenous pro- and anti-aggregatory factors derived from the whole animal (Born & Kratzel, 1981). Curiously, although the inhibitory properties of ET-1 on platelets have been explored in several animal species, there is, to our knowledge, no literature on this property of ET-1 in the murine model. Moreover, bradykinin (BK) is also a well-known stimulant of prostacyclin (PGI_2) and nitric oxide (NO) from vascular endothelial cells following activation of either B_1 or B_2 receptors (D'Orléans-Juste *et al*., 1989). Bradykinin has been shown to inhibit platelet aggregation in the dog (Imai & Matsubura, 1980) and the mouse (Hasan *et al*., 1996). It is therefore somewhat surprising that the receptors involved in anti-aggregatory properties of kinins have never been explored in this *ex vivo* murine model.

On the other hand, the interactions between vasoactive peptides and systemically-originated cyclo-oxygenase metabolites in a murine model of platelet aggregation *ex vivo* remained unexplored. Rosenblum *et al*. (1983) demonstrated that endogenous eicosanoids generated by intraplatelet cyclo-oxygenase pathways efficiently interfere with murine platelet aggregation in *in vitro* conditions. Interestingly, there is recent evidence suggesting the presence of constitutively expressed cyclo-oxygenase-2 (COX-2) in the mouse (Langenbach *et al*., 1998; Wallace, 1999).

The present study therefore proposes to firstly establish a pharmacological murine model to study the influence of

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vasoactive peptides, namely endothelin-1 and bradykinin, on *ex vivo* platelet aggregation. Secondly, the contribution of nitric oxide, COX-1 and COX-2 as well as prostacyclin will be assessed in the previously mentioned phenomenon. We will monitor the putative contribution of COX-1 and COX-2 with indomethacin and a selective inhibitor of the later isoform, NS-398 (Futaki *et al*., 1994). Furthermore, the eicosanoid involved in the inhibition of platelet aggregation induced by endothelin-1 and bradykinin will be characterized with tranylcypromine, a prostacyclin synthase inhibitor (Rosenblum & El-Sabban, 1978; Toda *et al*., 1982).

On the other hand, Offermanns *et al*. (1997) have shown a U46619 but not a thrombin-induced aggregation of murine platelets *in vitro* derived from animals knocked out for the $G\alpha_q$ subunit. It is known that platelets solely contain the $G\alpha_q$ subunit which subsequently activates phospholipase C in those cell fragments (Milligan *et al*., 1993). By that particular study, Offermanns *et al*. (1997) highlighted the usefulness of genetically-modified murine models in the understanding of platelet function *in vitro* as well as of the aetiology of thromboembolism.

Interestingly, it has recently been demonstrated that full knockout of the B_2 receptor gene for BK abolishes immune complex-induced peritoneal extravasation and prostaglandin E_2 release triggered by the nonapeptide in that animal (Samadfam *et al*., 2000). Whether the knockout of kinin receptors involved in the release of endothelial factors which interfere with platelet function is applicable to *ex vivo* monitoring of aggregation in the mouse, remains to be determined. The final part of this study will therefore address the role for kinin receptors in the *ex vivo* anti-aggregatory properties of bradykinin in homozygous B_2 knockout mice initially developed by Borkowski *et al*. (1995).

Our study suggests that ET-1 and BK inhibit platelet aggregation in the mouse *via* ET_B and B_2 receptors, respectively. For both agonists to induce that particular response, the contributions of COX-2 and consequently PGI_2 are required. In addition, BK but not ET-1, requires the concomitant release of nitric oxide and prostanoid to induce its inhibitory properties on *ex vivo* ADP-dependent platelet aggregation in the mouse.

Methods

The C57BL/6 B_2 receptor gene knockout mice were initially supplied by Dr Howard Chen (Merk, Rahway, U.S.A.) and are now routinely bred in our institution (Université de Sherbrooke). These mice are kept in the same normal conditions as their wild-type littermates of the identical genetic background (C57BL/6).

Male C57BL/6 mice or B_2 knockout animals (25–30 g) were anaesthetized with ketamine/xylazine (74/9.3 mg kg^{-1}, intramuscular). The left jugular vein and right carotid artery were canulated with polyethylene catheters (PE-10) for drug administration or continuous measurement of the mean arterial blood pressure (MAP) and blood collection, respectively. Changes in the MAP (mmHg) were measured with a pressure transducer (Staham, Model P23 A) and recorded on a Grass physiograph (Model 79). Blood (1.5 ml) was collected from two mice in a heparinized eppendorf (15 u ml^{-1}) *via* the carotid artery, 5 min after i.v. administration of ET-1 (0.01–1 nmol kg^{-1}), IRL-1620 (0.01–1 nmol kg^{-1}), BK (0.01–100 nmol kg^{-1}) or desArg^{9}bradykinin (100 nmol kg^{-1}). In another series of experiments, the ET_A or ET_B receptor antagonists, respectively, BQ-123 (1 mg kg^{-1}) or BQ-788 (0.5 mg kg^{-1}) were injected 5 min before each agonist. Separate groups of animals were treated with indomethacin (10 mg kg^{-1}), a non-selective COX inhibitor, NS-398, a COX-2 inhibitor, tranylcypromine, a prostacyclin synthase inhibitor, or L-NAME (10 mg kg^{-1}), a nitric oxide synthase inhibitor, 20 min before ET-1 injection (0.1 nmol kg^{-1}) or BK injection (10 nmol kg^{-1}).

Platelet-rich plasma (PRP) was obtained from blood samples by low-speed centrifugation (1100 r.p.m. for 12 min at room temperature) and diluted to obtain an average count of 500×10^9 platelets l^{-1} for an optimal adenosine diphosphate (ADP)-induced aggregation, as previously reported (Offermanns *et al*., 1997). Platelet-poor plasma (PPP) was prepared by centrifuging the whole blood sample at a higher speed (15,000 r.p.m. for 1 min at room temperature). PPP was used to calibrate the aggregometer at 100% transmission (Chronolog, Harvertown, U.S.A.) and platelet aggregation, from 400 μl samples (200 μl PRP and 200 μl saline), was monitored at 37°C in an aggregometer which measures the percentage of variation in light transmission produced through the cuvette following injection of ADP (0.5–10 μM; final concentrations).

ET-1 and IRL-1620 were purchased from American Peptide Company (Sunnyvale, CA, U.S.A.). BQ-123 and BK were synthesized in our laboratory. BQ-788 was purchased from Peptides International (Louisville, KY, U.S.A.), NS-398 from Biomol (Plymouth Meeting, PA, U.S.A.), ADP and tranylcypromine from Sigma (St. Louis, MO, U.S.A.). All agents were prepared in phosphate-buffered saline (PBS, pH 7.4, Sigma), except BQ-123 and BQ-788 which were previously dissolved in PBS + 20% DMSO (dimethyl sulphoxide from Fisher) to obtain 10 mg ml^{-1} stock solutions, and indomethacin, which was previously dissolved in PBS + 30% Trisma-base (0.2 M) and prepared fresh daily to obtain 10 mg ml^{-1} stock solution. Data are expressed as mean ± s.e.mean of n experiments. Statistical analyses were performed by Student's t-test. $^{***}P<0.001$; $^{**}P<0.01$; $^{*}P<0.05$.

Results

ADP induces a concentration-dependent aggregation of platelets in C57BL/6 mice

Platelet count in PRP was established at $977 \pm 63.2 \times 10^9$ platelets l^{-1} ($n=4$). In order to avoid fluctuations in platelet numbers, all experiments were performed in PRP diluted with saline 0.9% to obtain an average of 500×10^9 platelets l^{-1} (for optimal platelet aggregation).

In these conditions, ADP (0.5, 1, 5 and 10 μM) induced a concentration-dependent aggregation with a maximal response over 50% of aggregation, reached at 5 μM of ADP, when compared to control PPP (9.0 ± 3.2%, $n=4$; 23.5 ± 7.5%, $n=4$; 51.8 ± 2.1%, $n=14$; 51.1 ± 6.7%, $n=4$, respectively).

Ex vivo *murine platelet aggregation is inhibited by endothelin and bradykinin*

To monitor the platelet aggregation inhibitory properties of ET-1 and BK administered i.v. in C57BL/6 mice, the cells were routinely aggregated with a concentration of 5 μM ADP.

Figure 1 illustrates typical traces of PRP aggregated by ADP (Figure 1A,D), in addition to the inhibition of the phenomenon by i.v.-administered ET-1 (Figure 1C) or BK (Figure 1F). Furthermore, both ET-1 and BK had no direct effect on platelet aggregation induced by ADP (5 μM) *in vitro* (in presence of endothelin-1: 47.2±3.1%, $n=4$; in presence of bradykinin: 49±1.4%, $n=4$) (Figure 1B,E). In another series of experiments, the inhibitory effects were monitored on PRP derived from blood samples drawn 5 min, 30 min and 60 min following the i.v. administration of either ET-1 or BK. For both peptides, the inhibition of platelet aggregation was marked at 5 min ($P<0.05$) and was abolished at the 30-min and 60-min time points (ET-1 (0.1 nmol kg^{-1}); 17.0±3.7, 52.7±6.8 and 49.0±1.7%; BK (10 nmol kg^{-1}): 19.0±3.6, 56.7±9.4 and 50.0±2.1%, $n=3$).

Figure 2A illustrates the dose-dependent inhibition of *ex vivo* platelet aggregation triggered by systemic administration of endothelin-1 (0.01–1 nmol kg^{-1}) or IRL-1620 (0.01–1 nmol kg^{-1}) (control: 51.8±2.1%, $n=14$; in presence of the highest dose of IRL-1620 (1 nmol kg^{-1}): 18.1±5.3%, $n=4$). Doses of ET-1 higher than 1 nmol kg^{-1} induced a 50% mortality rate in wild-type animals. Furthermore, the selective ET_B antagonist, BQ-788 (0.5 mg kg^{-1}), but not BQ-123 (1 mg kg^{-1}), abolishes the inhibitory properties of endothelin-1 (Figure 3).

Finally, administration of bradykinin (1–100 nmol kg^{-1}), but not desArg^{9}bradykinin, a B_1 selective agonist (100 nmol kg^{-1}), induced a dose-dependent inhibition of platelet aggregation in the C57BL/6 mouse (Figure 2B).

Role of NO and prostanoids in the anti-aggregatory properties of endothelin-1 and bradykinin

In another series of experiments, pretreatment of C57BL/6 mice with indomethacin, NS-398 or tranylcypromine (TCP) (10 mg kg^{-1}) abolished the inhibitory properties of ET-1, whereas it required the concomitant administration of the COX or PGI_2 synthase inhibitors and a NOS inhibitor (L-NAME; 10 mg kg^{-1}) to fully impede the inhibitory properties of bradykinin (Figure 4).

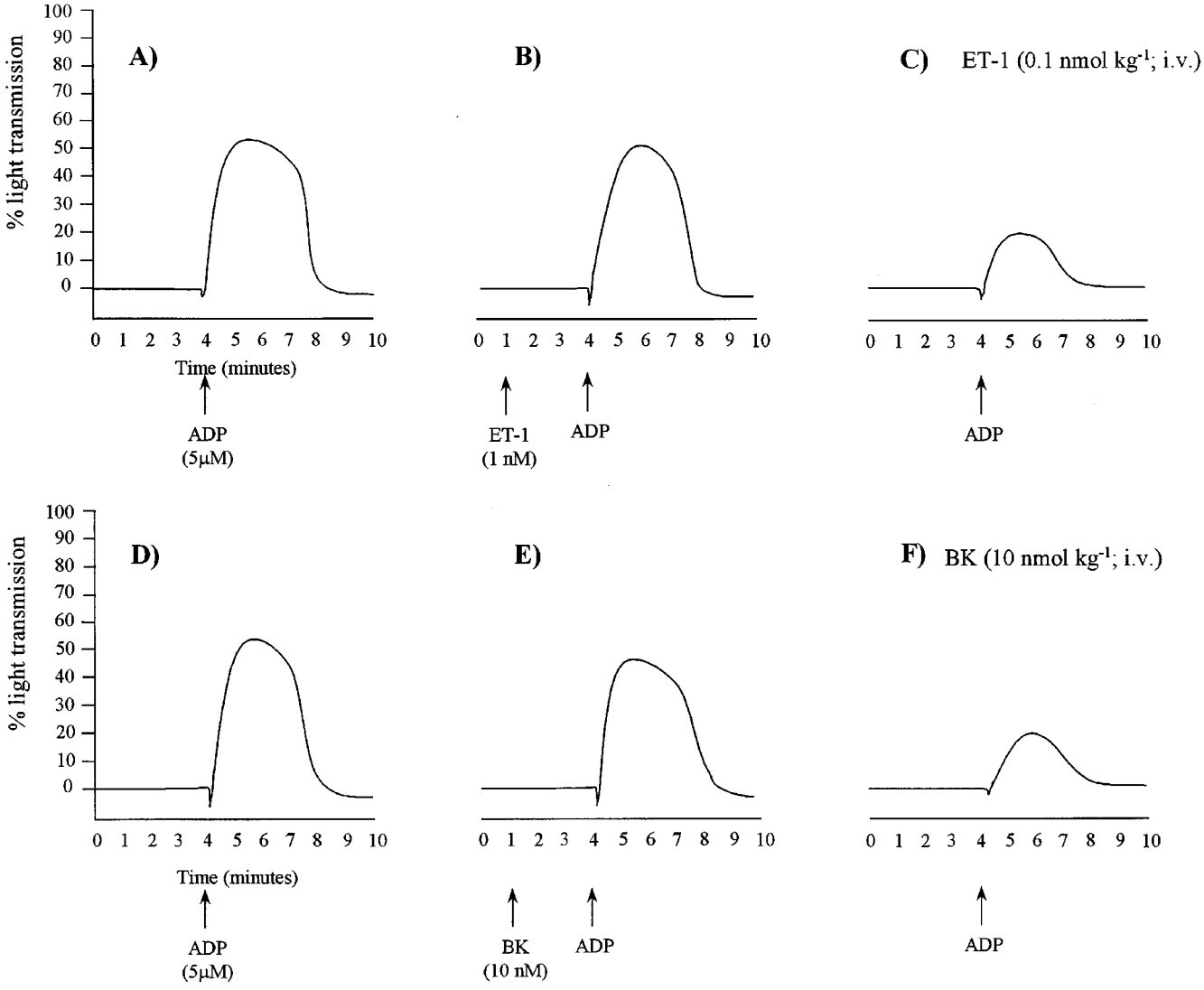

Figure 1 Typical traces of ET-1 and BK-induced inhibition of ADP-dependent aggregation of platelets *ex vivo*. Each peptide was administered either directly on platelets (B,E) or intravenously (C,F). Each trace is representative of 5–7 different experiments.

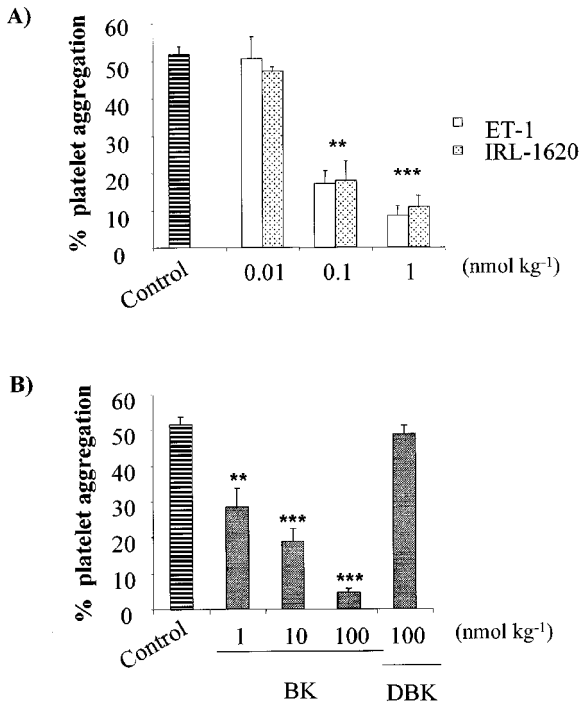

Figure 2 ET-1 and the ET_B agonist, IRL-1620, induces a dose-dependent inhibition of ADP-induced platelet aggregation (A). Administration of BK, but not desArg^9-BK (DBK), induces a dose-dependent inhibition of platelet aggregation (B). Each bar represents the mean ± s.e.mean of 4–7 experiments. $^{**}P<0.01$; $^{***}P<0.001$.

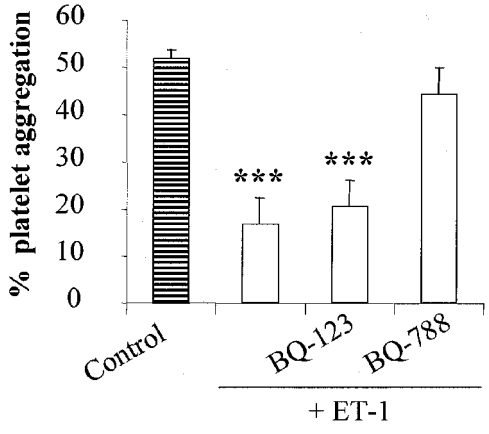

Figure 3 The ET_B antagonist, BQ-788 (0.5 mg kg^{-1}), but not the ET_A antagonist, BQ-123 (1 mg kg^{-1}), reverses the inhibitory effect of ET-1 (0.1 nmol kg^{-1}) on *ex vivo* ADP-induced platelet aggregation. Each bar represents the mean ± s.e.mean of 4–9 experiments. $^{***}P<0.001$.

Administration of indomethacin or indomethacin + L-NAME did not affect the *in vitro* aggregatory properties of ADP *per se* (control: 51.8 ± 2.1%, $n=4$; + Indo: 43.1 ± 4.3%, $n=4$; + Indo and L-NAME: 53.5 ± 6.3%, $n=4$). Furthermore, NS-398 and TCP were also devoid of direct effects on the ADP-induced aggregation of platelets (results not shown).

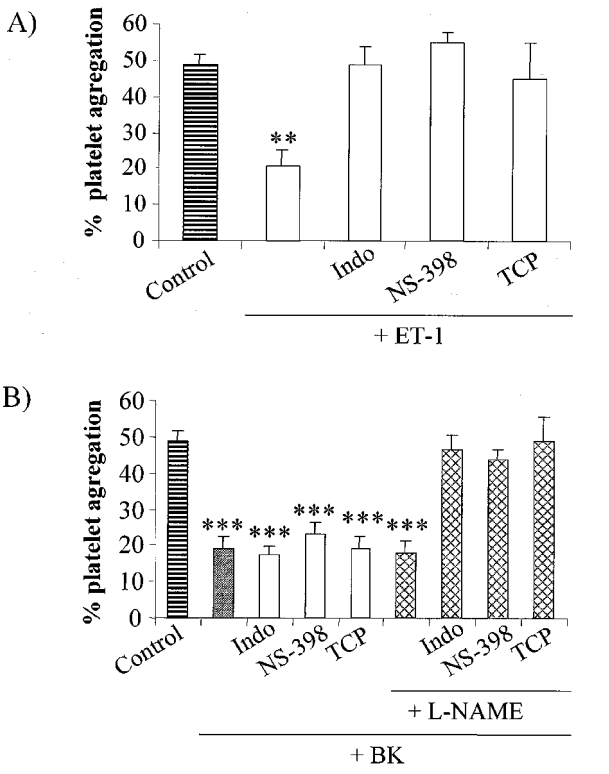

Figure 4 Indomethacin, NS-398 or tranylcypromine (TCP) (10 mg kg^{-1}) abolishes the anti-aggregatory effect of ET-1 (0.1 nmol kg^{-1}) (A), whereas only the concomitant administration of the cyclo-oxygenase or PGI_2 synthase inhibitors (10 mg kg^{-1}) and a nitric oxide synthase inhibitor L-NAME (10 mg kg^{-1}) is required to reduce the inhibitory properties of BK (10 nmol kg^{-1}) (B). Each bar represents the mean ± s.e.mean of 4–7 experiments. $^{**}P<0.01$; $^{***}P<0.001$.

Inhibitory properties of BK in B_2 (−/−)knockout mice

Firstly, ADP (5 μM) was as efficient to induce aggregation of platelets derived from B_2 (−/−) knockout mice (48 ± 5.4%, $n=6$) (Figure 5) as from wild-type C57BL/6 littermates (Figure 2B). In B_2 knockout mice, both bradykinin (1–100 nmol kg^{-1}; only 10 nmol kg^{-1} is shown) and desArg^{9}bradykinin (100 nmol kg^{-1}) were inefficient in interfering with the ADP-induced platelet aggregation *ex vivo*, whereas ET-1 (0.1 nmol kg^{-1}) triggered a significant inhibition of the same phenomenon (Figure 5).

Opposite haemodynamic effects of endothelin-1 or bradykinin in the anaesthetized mouse

Figure 6 illustrates the pressor or depressor responses to ET-1, IRL-1620 and bradykinin in C57BL/6 mice, whereas endothelin induces a dose-dependent increase of blood pressure at the same doses used to inhibit platelet aggregation *ex vivo*. IRL-1620 also induced a dose-dependent pressor response, whereas BK triggered a dose-dependent hypotension in the same wild-type animals. Furthermore, the hypotensive response to BK was abolished in B_2 knockout mice (results not shown).

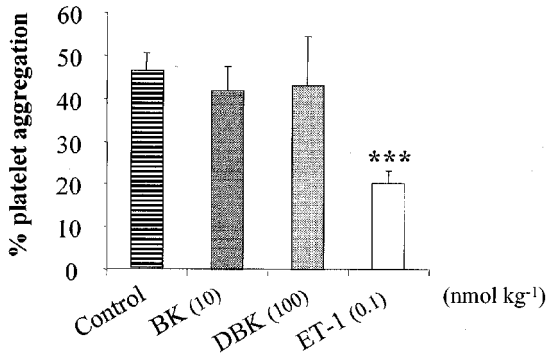

Figure 5 In B_2 knockout mice, BK and desArg^9-BK (DBK), but not ET-1, were inefficient to influence ADP-induced platelet aggregation. Each bar represents the mean ± s.e.mean of 4–9 experiments. ***$P<0.001$.

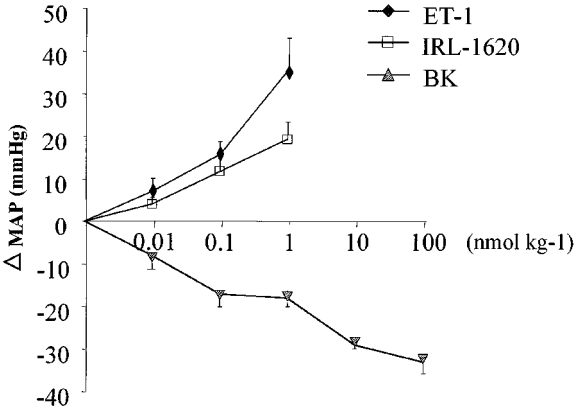

Figure 6 BK triggers a dose-dependent hypotension, whereas endothelin and IRL-1620, a selective ET_B receptor agonist, cause a dose-dependent increase in mean arterial blood pressure (MAP) in the anaesthetized mouse. Each bar represents the mean ± s.e.mean of 8–14 experiments.

Discussion

There is an increasing body of literature describing altered pharmacological and physiological phenotypes in genetically-manipulated mice. Such alterations have been documented, for example, in B_2 receptor knockout mice (salt-induced hypertension (Alfie *et al*., 1996)), in eNOS knockout animals (systemic hypertension (Huang *et al*., 1995)) and in a $G\alpha_q$ knockout murine model (thromboembolytic refractoriness (Offermanns *et al*., 1997)).

Considering the current literature on transgenic mice, particularly devoted to the study of platelet function *in vitro* (Thomas *et al*., 1994; Johnson *et al*., 1998), we established a murine model of *ex vivo* platelet aggregation, which allows the study of the influence of systemically-administered vasoactive peptides. In using this model, we found that both ET-1 and BK inhibit ADP-induced platelet aggregation *ex vivo via* ET_B and B_2 receptors, respectively, in the C57BL/6 mouse. The inhibitory properties of endothelin-1 are totally dependent of the release of endogenous eicosanoids in the murine model, in accordance with previous reports in the anaesthetized rabbit as well as in the dog (McMurdo *et al*., 1993; Herman *et al*., 1989a,b).

Interestingly, the concomitant inhibition of both cyclo-oxygenase and nitric oxide synthase is required to interfere with the inhibitory properties of bradykinin, as treatment with only one of those two inhibitors is insufficient to alter the *ex vivo* platelet aggregation induced by ADP in that animal species. The contribution of both cyclo-oxygenase and nitric oxide in the bradykinin-induced inhibition of platelet aggregation has already been reported in an *in vitro* model of porcine aortic endothelial cells and PRP obtained from human or rabbit blood (Radomski *et al*., 1987).

Our results also suggest that PGI_2 is the sole prostanoid involved, as tranylcypromine abolished the inhibitory properties of both ET-1 and BK in the mouse. The PGI_2-dependent-anti-aggregatory properties of ET-1 in that species are similar to what has been observed in the rabbit and the dog (McMurdo *et al*., 1993; Herman *et al*., 1989a,b).

On the other hand, COX-2 is now known to be constitutively expressed in several tissues (neuron, trachea epithelial cells, *macula densa* of the kidney) (Wallace, 1999). Interestingly, in the present study, we have highlighted the pivotal role of COX-2, since both indomethacin and NS-398 abolished the inhibitory properties of ET-1 and BK (+ L-NAME) on platelet aggregation. It may also be concluded that the contribution of COX-2, demonstrated in the mouse in the present study, is in accordance with McAdam *et al*. (1999) who recently reported that a significant portion (>80%) of prostacyclin production in healthy humans occurs *via* COX-2. This particular observation raises the concern that inhibition of endothelial prostacyclin synthesis, while sparing platelet production of TxA_2, may predispose a susceptible individual to a thrombotic event or exacerbation of hypertension.

The present study also shows that the platelet inhibitory properties of both endothelin-1 and bradykinin are not due to mechanical response of the underlying smooth muscle of those agonists, as they have opposite effects on the mean arterial blood pressure of these animals. Endothelin-1 and IRL-1620 administered intravenously both induced pressor responses, confirming the contribution of both ET_A and ET_B receptors in the vasoconstrictive effects of endothelins in the mouse (Berthiaume *et al*., 2000). In contrast, bradykinin induces a hypotension solely due to B_2 receptor activation (Alfie *et al*., 1996). We therefore suggest that the endothelial cell is the effector for the ET-1 and BK-induced release of eicosanoids and nitric oxide (only for the later peptide) which in turn interfere with platelet aggregation *ex vivo* in the mouse.

Worthy of notice, albeit the inhibitory process is triggered *in vivo*, the refractory phase to a pro-aggregatory factor, such as ADP, appears long-lasting (over 20 min) when taking into account the time required to obtain blood samples, to separate PRP from PPP and, finally, to assess the light refraction ratios between those two preparations. However, the inhibitory properties of both peptides administered intravenously are reversible, since no inhibition of ADP-induced aggregation was detected when the platelets were collected 30 min after the administration of ET-1 or BK.

Interestingly, in our hands, the use of B_2 knockout mice suggests that the anti-aggregatory properties of the systemically-administered bradykinin is mediated solely by B_2

receptors and that in these particular conditions, there is no upregulation of B_1 receptor-dependent influences on *ex vivo* platelet behaviour. Several responses to bradykinin are abolished in B_2 knockout mice, such as endothelium-dependent vasodilation (Berthiaume *et al*., 1997), plasma extravasation (Samadfam *et al*., 2000) or inhibition of platelet aggregation (present study). Nonetheless, the lack of B_2 receptors does not alter the overall capacity of these animals to generate sufficient endogenous factors to inhibit platelet aggregation in these genetically modified animals when challenged by other agonists than kinins, as illustrated with ET-1 in the present study.

In summary, this is to our knowledge the first report demonstrating the inhibitory properties of ET-1 and BK *via* ET_B and B_2 receptors, respectively, in a model of *ex vivo* platelet aggregation in the mouse. This system will now allow study of the contribution of endogenous factors on the *ex vivo* aggregatory properties of platelets in genetically-modified murine models. Such cell–cell-derived humoral modulations can be advantageously assayed in the present model, for example, in selective knockout of GIIbIIIa (Hodivala-Dilke *et al*., 1999), eNOS (Huang *et al*., 1995), iNOS (Wel *et al*., 1995), peptide receptors (B_2, heterozygous (+/−), ET_A or ET_B (Borkowski *et al*., 1995; Clouthier *et al*., 1998; Hosoda *et al*., 1994) and/or adhesion molecules (Law *et al*., 1999)) in the genetically-manipulated mouse.

We conclude that this model will be useful to pharmacologically identify the receptors for several vasoactive peptides involved in the alteration of platelet function and, in addition, may prove to be valuable to study phenotypic alterations of platelet function *ex vivo* in genetically-modified animals.

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