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Contents

Editorial

Welcome to the July 2003 issue of *The* Bulletin!

Our review article for this issue has been written by Dr Aleksandar Jovanovic of the Tayside Institute of Child Health at Dundee. Dr Jovanovic provides a comprehensive overview of recent resaerch into the function of sarcolemmal K_{ATP} channels and their potential role in cardioprotection.

We are pleased to include a report on the recent BSCR meeting at the University of Glasgow entitled "*Molecular Therapy for Cardiovascular Disease*". The report has been written by the organisers, Drs Andrew Baker and Sarah George and the abstracts presented at the meeting are also included. On behalf of the Society we wish to express our gratitude to the organisers for arranging an extremely successful and enjoyable meeting.

We can look forward to another BSCR meeting in Scotland this year when we visit Edinburgh in September. The Autumn meeting on "*Oxidative Stress: from Measurement to Management*" is being organised by Gillian Gray and colleagues and further details can be found on the back cover.

Finally, we bring you the latest details of grants awarded to researchers in the Cardiovascular field, by the Wellcome Trust.

Helen Maddock and Nicola Smart

Sarcolemmal K_{ATP} channels

by
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Introduction

Two decades ago, Noma (1983) uncovered a novel K^+ channel in cardiac ventricular myocytes termed ATP-sensitive K^+ (K_{ATP}) channel which was gated directly by intracellular ATP, i.e. in an ATP-free environment a K^+-selective conductance was readily active to be inhibited by intracellular, micromolar levels, of ATP. From then, numerous studies have shown that similar types of K^+ channels are present in different tissue and cell types, including pancreatic β-cells, skeletal muscle cells, vascular and other smooth muscle cells, neuronal cells, endothelial cells, and renal epithelial cells. It is generally accepted that the defining attributes of K_{ATP} channels are inhibition by ATP and sulfonylurea drugs and stimulation by nucleotide diphosphates and K^+ channel-opening drugs (for detailed and general reviews on basics of K_{ATP} channels see Yokoshiki et al., 1998; Seino, 1999; Baukrowitz and Fakler, 2000). Although these characteristics are shared with K_{ATP} channels in different tissues, differences in properties among pancreatic, sarcolemmal, and vascular smooth muscle cells do exist, suggesting differences in structure, regulation and function between K_{ATP} channels in different tissues. Probably the most crucial step in understanding these differences was molecular cloning of K_{ATP} channels (Inagaki et al., 1995; 1996; Isomoto et al., 1996). This work suggested that native K_{ATP} channels are a complex of regulatory protein(s) containing the sulfonylurea receptor (SUR subunit) and an inwardly-rectifying K^+ channel (Kir) serving as a pore-forming subunit. This and later research (Shyng and Nichols, 1997) suggested that the molecular structure of K_{ATP} channels is a heteromultimeric assembly of Kir6.2 with SUR1 (SUR1/Kir6.2, pancreatic type), Kir6.2 with SUR2A (SUR2A/Kir6.2, cardiac type), and Kir6.1 with SUR2B (SUR2B/Kir6.1, vascular smooth muscle type). Structure-function studies on cardiac K_{ATP} channels have identified many of the amino-acids controlling nucleotide-depending channel gating and second messenger modulation (Tucker et al., 1997; Trapp et al., 1998; Lin et al., 2000; Babenko and Bryan, 2002; Cukras et al., 2002). In addition, the most recent studies have suggested that the cardiac type of K_{ATP} channels could be composed of more proteins than just Kir6.2 and SUR2A subunits (Carrasco et al., 2001; Crawford et al., 2002a, b; for details see below).

In contrast to the advanced understanding of the structure and regulation of K_{ATP} channels, less is known about the functional consequences of activation of cardiac K_{ATP} channels. There are several difficulties in determining the effects of activation of these channels upon cardiac function, most importantly, perhaps, is the possible existence of non-sarcolemmal, mitochondrial, K_{ATP} channels in cardiomyocytes (considering that the structure of mitochondrial K_{ATP} channels is still unknown and their existence still matter of vigorous debate, the term putative mitochondrial K_{ATP} channel is used in later text). The activation of putative mitochondrial K_{ATP} channels by K_{ATP} channel openers (the nature of K^+ conductance in mitochondria was also tested by K_{ATP} channel blocking drugs), has been proposed to affect mitochondrial membrane potential, mitochondrial and Ca^{2+} homeostasis and cardiac resistance to different metabolic stresses, including ischaemia and hypoxia (Garlid et al., 1997; Liu et al., 1998; Holmuhamedov et al., 1998; 1999). The drugs used in these studies also modulate the activity of K_{ATP} channels, composed of Kir6.2/SUR2A subunits and known originally as cardiac type K_{ATP} channels, Inagaki et al., 1996; the term sarcolemmal K_{ATP} channel was used later to distinguish between K_{ATP} channels expressed in sarcolemma and mitochondria, see Gross and Fryer, 1999). As such, it was almost impossible to separate the functional consequences of activation of putative

mitochondrial from those of activation of sarcolemmal K_{ATP} channels (for deeper understanding of this particular issue see Hu et al., 1999; Ovide-Bordeaux et al., 2000; Ozcan et al., 2002; Suzuki et al., 2003). However, research using recombinant channel subunits and mice with genetically disrupted sarcolemmal K_{ATP} channels successfully shed a little more light on the functional consequences of changes in activity of sarcolemmal K_{ATP} channels (Jovanovic et al., 1998; Jovanovic and Jovanovic, 2001a; Crawford et al., 2002b; Suzuki et al., 2002; 2003).

The purpose of this short review is to bring to the reader's attention some research related to sarcolemmal K_{ATP} channels of recent years, shedding new light on the structure, regulation and function of sarcolemmal K_{ATP} channels.

Structure and regulation of sarcolemmal K_{ATP} channels

It is now generally accepted that the sarcolemmal K_{ATP} channel is a heteromultimeric protein complex composed of Kir6.2 or Kir6.1 and SUR2A (see above). It has been suggested that the pore-forming inwardly-rectifying K^+ channel core of the sarcolemmal K_{ATP} channel may be either Kir6.2 or Kir6.1 (Cui et al., 2001). While it is certain that Kir6.2/SUR2A reconstitute the channel with electrophysiological properties similar to those of native sarcolemmal K_{ATP} channels (Inagaki et al., 1996), it is not entirely clear what role Kir6.1, as a pore-forming subunit, might play in the heart. So far, major functional studies suggested that the main form of sarcolemmal K_{ATP} channels is composed of Kir6.2 and SUR2A subunits (Inagaki et al., 1996; Babenko et al., 1998). However, it should be pointed out that data have been provided to suggest that Kir 6.1 and Kir 6.2 may co-assemble to produce functional channels (Cui et al., 2001). There is evidence that chronic exposure to ischaemia increases expression of SUR2A and Kir6.1, but not Kir6.2, subunits, suggesting that ischaemic heart may predominantly express the Kir6.1/SUR2A instead of Kir6.2/SUR2A channel type (Akao et al., 1997; Melamed-Frank et al., 2001). In recombinant systems Kir6.1/SUR2A channels have significantly lower single channel conductance than Kir6.2/SUR2A (Kondo et al., 1998); however no electrophysiological evidence has so far been provided to support the hypothesis that the Kir6.1/SUR2A combination is present in the sarcolemma of ischaemic heart. It should be also stated that more recent studies suggest that the level of the SUR2A subunit is the major factor controlling the number of sarcolemmal K_{ATP} channels. This is due to the presence of Kir6.2 in excess over the SUR2A subunit in cardiomyocytes (for more detailed justification of this conclusion see Ranki et al., 2001; 2002a,b). Thus, whether Kir6.1 is a component of sarcolemmal K_{ATP} channels *in vivo* remains controversial (see also Seharaseyon et al., 2000). Beside SUR2A, cardiac tissue also expresses the SUR2B subunit, but the consensus view is that SUR2A serves as the only regulatory subunit of sarcolemmal K_{ATP} channels since co-assembly between SUR2A and SUR2B seems to be impossible (Giblin et al., 2002). Structurally, Kir 6.2 belongs to the family of the two-membrane spanning domain, inwardly rectifying K^+ channels while the SUR2A subunit belongs to the family of ATP-binding proteins and is assumed to possess 17 putative transmembrane regions (for the molecular structure of in details Kir6.x and SURx see Tanemoto et al., 2001). However, more recent evidence suggests that, *in vivo*, the sarcolemmal K_{ATP} channel protein complex is composed of more proteins than Kir6.2 and SUR2A subunits (Carrasco et al., 2001; Crawford et al., 2002a,b). It has been proposed that adenylate kinase (AK; enzyme catalysing AMP+ATP<=>2ADP), creatine kinase (CK; enzyme catalysing ADP+phosphocreatine<=>ATP+creatine) and the muscle form of lactate dehydrogenase (M-LDH; enzyme catalysing pyruvate+NADH<=>lactate+NAD) are components of the sarcolemmal K_{ATP} channel protein complex (**Figure 1**). Compelling evidence has been provided to suggest that CK directly physically interact with the SUR2A subunit *in vivo* (Crawford et al., 2002a). On the other side, M-LDH physically associates with both SUR2A and Kir6.2 subunits, interacting via its C- and N-termini respectively (Crawford et al., 2002b), while interaction of subunit(s) with AK remain(s) to be determined. ATP acts on K_{ATP} channel subunits (both Kir6.2 and SUR2A) to close the channel while ADP and lactate have been reported to directly open the channel (Noma, 1983, Lederer and Nichols, 1989; Han et al., 1993; Terzic et al., 1994; Tucker et al., 1997; 1998; Crawford et al., 2002b). In this regard, it seems that the physical attachment of AK, CK and M-LDH to Kir6.2/SUR2A allows tight control of levels of K_{ATP} channel ligands, (ATP, ADP and lactate) within the microenvironment surrounding the channel. In turn, this could efficiently control K_{ATP} channel activity. Consequently, while ATP alone close the channel, the concomitant presence of AMP (AMP itself does not affect the channel opening, Elvir-Mairena et al., 1996) and ATP or creatine (creatine itself does not affect the channel opening, Carrasco et al., 2001) and ATP or pyruvate (pyruvate itself does not affect the channel opening, Crawford et al., 2002b) and NADH (NADH itself does not affect the channel opening, Crawford et al., 2002b)

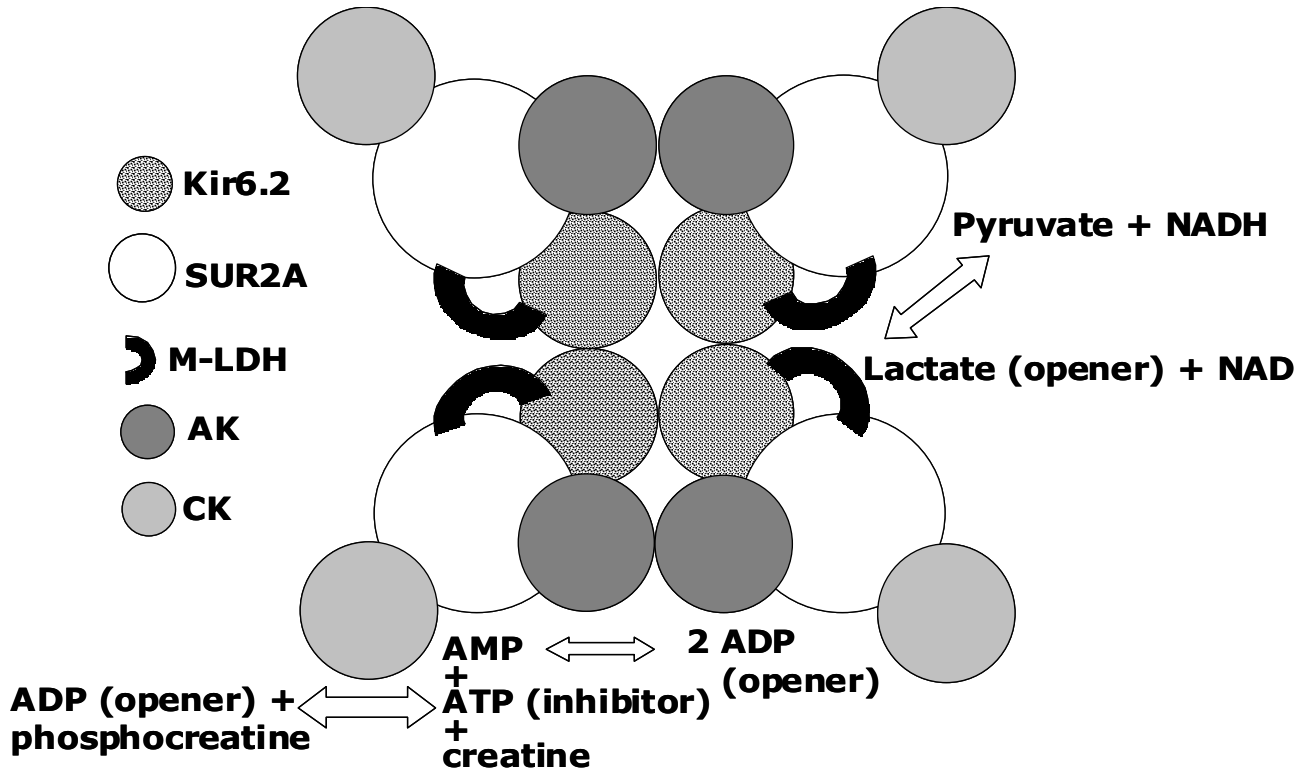

Figure 1. Schematic representation of a possible protein composition of sarcolemmal K_{ATP} channels *in vivo*. Reaction catalysed by proteins composing this channel with enzymatic activity are depicted as well. This scheme is drawn based on work described Inagaki et al., 1995; 1996; Shyng and Nichols, 1997; Carrasco et al., 2001; Crawford et al., 2002a,b (see list of references). Opener = compound that activates sarcolemmal K_{ATP} channels; Inhibitor = compund that closes sarcolemmal K_{ATP} channels.

open the channel (**Figure 1**). Furthermore, AK-, CK- and M-LDH share substrates, which potentially creates a very fine "tuner" to regulate sarcolemmal K_{ATP} channel activity. For example, AK-mediated opening of the K_{ATP} channels (AMP plus ATP induces opening of K_{ATP} channels, Elvir-Mairena et al., 1996; Carrasco et al., 2001) could be reversed by CK activity (ADP plus phosphocreatine induces closing of K_{ATP} channels, Bienengraeber et al., 2000; Carrasco et al., 2001; Crawford et al., 2002a). It is possible that, under physiological conditions, CK catalyses the production of ATP from phosphocreatine and ADP, maintaining high ATP/ADP ratio around the channel microenvironment and keeping the channel in a closed state despite the presence of AK (Carrasco et al., 2001; Crawford et al., 2002a). During severe metabolic stress in the heart, CK velocity dramatically decreases together with a decrease in the phosphocreatine/ATP ratio (Cave et al., 2000). Both of these processes would inhibit production of ATP (a decrease in CK velocity) and increase levels of ADP within the channel vicinity, catalysed by AK or even by CK itself (a decrease in the phosphocreatine/ATP ratio and an increase in creatine), activating sarcolemmal K_{ATP} channels (Carrasco et al., 2001; Crawford et al., 2002a). At the same time, under aerobic, physiological, conditions, intracellular levels of lactates would be very low as LDH activity produces pyruvate to be further utilised in mitochondria by pyruvate dehydrogenase. Under anaerobic conditions, pyruvate, NAD and H^+ can not be metabolised further and accumulate (see Kantor et al., 2001), leading to the M-LDH-catalysed lactate production within the microenvironment surrounding the channel. Lactate opens the channel even in the presence of high levels of ATP (Crawford et al., 2002b).

In addition to regulation by ATP, ADP and lactate, there other factors that may directly or indirectly affect the activity of sarcolemmal K_{ATP} channels. The activity of K_{ATP} channels may be regulated by non-ATP and non-ADP nucleotides (Lederer and Nichols, 1989; Jovanovic et al., 2001), changes in intracellular pH (Davies, 1990; Baukrowitz et al., 1999), the status of the cytoskeleton (Terzic and Kurachi, 1996), protein kinase C (Light et al., 2001), phosphatidylinositol-4,5-

bisphosphate (Hilgemann and Ball, 1996), and by the operative condition of the channel itself (Terzic et al., 1994). Disruption of actin cytoskeleton, intracellular acidification and phosphorylation state of K_{ATP} channels appear to be conditions that promote the opening of K_{ATP} channels (Terzic et al., 1994; Terzic and Kurachi, 1996; Baukrowitz et al., 1999). In general, it seems that the mechanism responsible for the channel opening under the above conditions relates to a decrease in the sensitivity of K_{ATP} channels towards ATP (Terzic et al., 1994; Terzic and Kurachi, 1996; Baukrowitz et al., 1999). In inside-out configuration patch clamp technique ~100 μM of ATP is sufficient to block the channel activity (Noma, 1983; Terzic et al., 1994) which is 50-100 times less then those physiologically found, and much lower than the ATP concentration in a living cardiomyocyte exposed to severe ischaemia (reviewed by Kantor et al., 2001). Thus, the closed state of the channel under physiological conditions could be explained by high intracellular ATP (and possibly by presence of other, non-ATP, inhibitory, nucleotides which role in the heart is not clear yet, for details see Jovanovic et al., 2001) while the opening of sarcolemmal K_{ATP} channels during metabolic stress could be due to several factors including decrease in ATP/ADP ratio, increase in lactate levels, decrease in intracellular pH, disruption of cytoskeleton and the action of protein kinase C (Noma, 1983; Terzic et al., 1994; Terzic and Kurachi, 1996; Baukrowitz et al., 1999; Carrasco et al., 2001; Crawford et al., 2002a,b). The exact mechanisms responsible for the sarcolemmal K_{ATP} channels opening *in vivo* is still to be fully investigated. What is generally accepted is that sarcolemmal K_{ATP} channels are closed in healthy heart and are opened during ischaemia (reviewed by Tanemoto et al., 2001).

Sarcolemmal K_{ATP} channels and cardioprotection

In the last decade, drugs that may inhibit and/or activate K_{ATP} channels have been used in order to determine the consequences of sarcolemmal K_{ATP} channel opening (reviewed by Jahangir et al., 2001; Gribble and Reiman, 2002). Channel inhibitors (sulfonylurea drugs (glybenclamide, tolbutamide), 5-hydroxydecanoic acid (5-HD) etc.) and activators (pinacidil, diazoxide, cromakalim etc.) were applied at different times during ischaemia, metabolic inhibition or hypoxia (in later text these injuries are termed metabolic stress) in a number of experimental models ranging from single cells to whole animals and even humans (Grover et al., 1990; Gross and Auchampach, 1992; Jovanovic et al., 1998; Ranki et al., 2001; Lee et al., 2002). In early days of K_{ATP} channel studies in the heart it was concluded that that the opening of sarcolemmal K_{ATP} channels protect the heart against metabolic stress, including myocardial infarction (Grover et al., 1990; Gross and Auchampach, 1992). However, this conclusion was challenged when it was uncovered that some K_{ATP} channel opening drugs may affect the membrane potential of the mitochondria, ascribed to the effect on putative mitochondrial K_{ATP} channels (Liu et al., 1998; 1999). Putative mitochondrial K_{ATP} channels were described for the first time in 1991 (Inoue et al., 1991) and in 1997 (Garlid et al., 1997) it was suggested that the opening of these channels, rather then sarcolemmal K_{ATP} channels, may mediate cardioprotection (see also Sato et al., 2000). This conclusion was largely based on the use of diazoxide and 5-HD as selective opener and antagonist of mitochondrial K_{ATP} channels, respectively (Garlid et al., 1997; Liu et al., 1998; 1999; Sato et al., 2000). However, more recent studies suggested that the effect of diazoxide and 5-HD on mitochondria is probably not associated with changes in mitochondrial K^+ conductance, which questions the involvement of mitochondrial K_{ATP} channels in cardioprotection (Ovide-Bordeaux et al., 2000; Ozcan et al., 2002; Hanley et al., 2002; Das et al., 2003). The fact that the structure of the putative mitochondrial K_{ATP} channel is as yet unknown precludes further research that would potentially define the significance and role of these channels. On the other hand, more recent work utilising selective antagonists of sarcolemmal K_{ATP} channels, recombinant channel proteins and mice that do not express sarcolemmal K_{ATP} channels provided compelling evidence that the activation of this ion channel is cardioprotective (Jovanovic et al., 1998; 1999; Jovanovic and Jovanovic, 2001a; Suzuki et al., 2002; 2003). This conclusion is based on the findings that: 1) coexpression of Kir6.2 with SUR2A in combination with K_{ATP} channel opener or M-LDH confers resistance against metabolic stress in otherwise stress-sensitive cells (Jovanovic et al., 1998; Crawford et al., 2002b); 2) in mice with genetically disrupted sarcolemmal K_{ATP} channel the heart is more susceptible to metabolic stress (Zingman et al., 2002); 3) K_{ATP} channel opener-mediated protection against metabolic stress is associated with the effect on cardiac membrane potential and with measurable sarcolemmal K_{ATP} channel opening (Jovanovic and Jovanovic, 2001b; Suzuki et al., 2002); 4) an increase in the number of sarcolemmal K_{ATP} channels increase cardiac resistance to metabolic stress and this is blockable by HMR 1098, a selective

antagonist of sarcolemmal K_{ATP} channels (Ranki et al., 2001; 2002b) and 5) HMR 1098 inhibits cardioprotection mediated by protein kinase C (Light et al., 2001). The mechanism underlying cardioprotection mediated by sarcolemmal K_{ATP} channels is still a matter of considerable discussion. Intracellular Ca^{2+} overload and depletion of energy stores underlie the cell injury and death that occur during metabolic stress. The opening of K_{ATP} channels reduces action potential duration, decreases Ca^{2+} influx (preventing increase in cytosolic Ca^{2+}) and hypercontracture, conserving energy stores and protecting the heart against injury (Jovanovic et al., 1998; Jovanovic and Jovanovic, 2001a,b; Crawford et al., 2002b; Suzuki et al., 2002). For now, this is believed to be how opening of sarcolemmal K_{ATP} channels mediate cardioprotection.

One of the important aspects of myocardium exposed to metabolic stress is appearance of arrhythmias. In theory, the activation of sarcolemmal K_{ATP} channels could be both pro-and anti-arrhythmogenic (see Remme and Wilde, 2000; Flagg and Nichols, 2001). Increased K^+ conductance shifts resting membrane potential towards K^+ equilibrium which should reduce ectopic pacemaker activity. On the other hand, activation of K_{ATP} channels accelerates the repolarisation of the action potential, reducing the refractory period of the cell, which may increase the probability of re-entrant arrhythmias (reviewed by Tristani-Firouzi et al., 2001). Research addressing whether the opening of sarcolemmal K_{ATP} channels induces arrhythmias provided inconclusive results so far. It has been reported that glybenclamide, a prototype antagonist of K_{ATP} channels, decreased the incidence of tachycardia and ventricular fibrillation (Kantor et al., 1990); however in some other studies the exact opposite was reported (Shigamatsu et al., 1995). Similarly, in certain studies, the opening of K_{ATP} channels has been shown to promote tachycardia and fibrillation (Billman et al., 1998) while in other studies, channel openers have been reported to reduce the frequency of arrhythmias (Das et al., 2001). In mice genetically lacking sarcolemmal K_{ATP} channels no shortening of action potential duration was observed during ischaemia or upon application of K_{ATP} channel openers (Suzuki et al., 2002). Conversely, in transgenic mice overexpressing K_{ATP} channels that are ~2 orders of magnitude less sensitive to inhibition by ATP, a decreased frequency of ectopic heart beats was observed suggesting that sarcolemmal K_{ATP} channels may reduce the frequency of early or delayed afterdepolarisation-induced ectopic beats (Koster et al., 2001). The peaked T wave and ST elevation is a characteristic finding on ECG in ischaemic heart and both are blocked by K_{ATP} channels blocking drugs, suggesting that the opening of K_{ATP} channels underlie these abnormalities in the repolarisation phase of ECG (Kubota et al., 1993; Kondo et al., 1996). However, these data should be cautiously interpreted since the effect of pinacidil, a K_{ATP} channel opener, on heart electrophysiology may be inhibited by antagonists of non-K_{ATP} channel K^+ conductance (Li et al., 2000). This highlights the point that ECG profile results from integration of multiple conductances, the inhibition of a current conducted by one type of ion channels may change the ECG profile without pinpointing the specific cause (see also Flagg and Nichols, 2001).

Although further research is clearly required to assess all clinical consequences of sarcolemmal K_{ATP} channel opening, it should be pointed out that there are clinical studies that would suggest overall cardioprotective outcome of K_{ATP} channel activation. In this regard, an increase in mortality due to cardiovascular causes among diabetic patients taking sulfonylurea drugs compared to patients treated with insulin or placebo was published more than 30 years ago (Klimt et al., 1970). This notion is also supported in a recent article by Garratt *et al* (1999) where it was reported that sulfonylurea drugs adversely affected outcome among diabetics undergoing direct angioplasty in the setting of acute myocardial infarction (Garratt et al., 1999). In this particular study, risk of death was found to be ~3 times higher in diabetics taking sulfonylurea drugs and these drugs were independently predictive of worse outcome. Thus, although the full consequence of sarcolemmal K_{ATP} channels opening has to be further studied, it is likely that the opening o these channels is beneficial for heart exposed to at least some forms of metabolic stress.

Ischaemic preconditioning and sarcolemmal K_{ATP} channels

In 1986 Murray et al. demonstrated, in anesthetized dogs, that a 40-min occlusion of a coronary artery results in 29% infarction of the myocardium served by this vessel (Murry et al., 1986). However, when that 40-min occlusion was preceded by four cycles of 5 min occlusion/5 min reperfusion infarction was only 7% of the risk zone. Thus, these

short episodes of ischaemia/reperfusion diminished the infarction by 75% although the total duration of ischaemia had increased from 40 to 60 min (Murray et al., 1986). This phenomena has been termed ischemic preconditioning and it is defined as the increased resistance to myocardial infarction that follows short sublethal periods of ischemia. Preconditioning's anti-infarct effects was shown to be independent of coronary blood flow and recruitment of coronary collaterals (Murry et al., 1986; Jennings et al., 1991). These findings suggested that some powerful naturally-occurring non-vascular endogenous mechanism underlies ischemic preconditioning (Cohen et al., 2000). In the early days of preconditioning research, it was shown that sulfonylurea drugs may block preconditioning which led to the conclusion that the opening of K_{ATP} channels is involved in preconditioning (Gross and Auchampach, 1992). However, based on results obtained with diazoxide and 5-HD and under the assumption that these drugs selectively target putative mitochondrial K_{ATP} channels some studies challenged this notion suggesting that mitochondrial, but not sarcolemmal, K_{ATP} channels mediate preconditioning (Liu et al., 1999; Ghosh et al., 2000). However, the selectivity and specificity of drugs targeting K_{ATP} channels have recently been questioned (Ovide-Bordeaux et al., 2000; Ozcan et al., 2002; Hanley et al., 2002; Das et al., 2003). At the same time, a recent study with mice lacking sarcolemmal K_{ATP} channels has revealed that ischaemic preconditioning can not be reproduced in these mice (Suzuki et al., 2002), which seems to be strong evidence in favour of the notion that sarcolemmal K_{ATP} channels are involved in ischaemic preconditioning. What activates these channels to mediate preconditioning is not yet clear. It has been reported that preconditioning may activate protein kinase C (PKC) which, in turn, may target and activate sarcolemmal K_{ATP} channels (Light et al., 2001). Apart from PKC there are other factors that could open the K_{ATP} channels including numerous enzymes and intracellular signalling factors (described in the previous section) that could be activated by ischaemic preconditioning. However, research addressing the opening of sarcolemmal K_{ATP} channels as a part of the mechanism underlying ischaemic preconditioning is in its early stages and there is still no conclusive evidence to define the signalling cascades which mediate the opening of sarcolemmal K_{ATP} channels *in vivo* in ischaemic preconditioning.

Conclusions and future

At the present stage of knowledge about sarcolemmal K_{ATP} channels it is probably accurate to conclude that these channels are 1) composed *in vivo* of more proteins than Kir6.2/SUR2A; it is likely that the majority of these proteins are enzymes that regulate concentration of K_{ATP} channel ligands (ATP, ADP and lactate) in the microenvironment surrounding the channel and, consenquently, regulate the activity of sarcolemmal K_{ATP} channels; 2) regulated by a complex interaction between intracellular signalling factors and cascades and not by nucleotides alone; 3) an important factor in regulating cardiac resistance to metabolic stress and are involved in the signalling pathway mediating ischaemic preconditioning.

However, there are various aspects of sarcolemmal K_{ATP} channels that need to be addressed in the future. Although it is appreciated that numerous factors may influence the K_{ATP} channel activity it is still unknown what underlies the channel opening *in vivo* in myocardium suffering from ischaemia and other types of metabolic insults. Although Kir6.2/SUR2A reconstitute basic properties of sarcolemmal K_{ATP} channels in cell lines natively devoid of these channels, recombinant K_{ATP} channels remain mostly closed throughout the metabolic challenge, which is not how sarcolemmal K_{ATP} channels behave *in vivo*. However, co-expression of Kir6.2/SUR2A/M-LDH creates a cellular phenotype that is more resistant to metabolic stress due to "on-time" opening of recombinant K_{ATP} channels suggesting that signalling pathways yet to be uncovered may play a role in cardioprotection afforded by these channels. Further research utilising a range of different experimental models is needed to define the mechanism underlying sarcolemmal K_{ATP} channel opening *in vivo* and also to fully understand the consequences of channels opening on cardiac function (not to mention need to resolve controversies about the effect of sarcolemmal K_{ATP} channel activation on cardiac rhythm).

There are probably several reasons why some questions related to sarcolemmal K_{ATP} channels are difficult to answer. One important reason is possibility of existence of non-sarcolemmal K_{ATP} channels in the heart (such as mitochondrial K_{ATP} channels) which makes questionable whether a particular effect is a consequence of activation of sarcolemmal K_{ATP} channels. A predominant strategy in addressing the function of sarcolemmal K_{ATP} was limited on use of K_{ATP} channel antagonists and activators. As with any

other drug, selectivity and specificity of these drugs are less then perfect which precludes drawing strong final conclusions. To overcome these difficulties use of transgenic animals lacking (already generated by Suzuki et al., 2002) and overexpressing sarcolemmal K_{ATP} channels (to be generated) is warranted. Repetition of different types of experiments addressing sarcolemmal K_{ATP} channels (whole heart and single cell models of ischaemia, myocardial infarction, ischaemic preconditioning etc.), that have been previously done on genetically non-modified animals, as well as experiments with new, original design, on mice lacking and overexpressing K_{ATP} channels would probably provide the answers to yet unanswered questions about regulation, function and role of sarcolammal K_{ATP} channels. Better understanding of sarcolemmal K_{ATP} channels would, potentially, help to develop a better therapeutic strategy against diseases associated with metabolic stress of the heart, including myocardial infarction and chronic ischaemic heart disease.

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Articles for *The* Bulletin

Would you like to write a Review or Laboratory Profile for the BSCR Bulletin? These articles provide an excellent opportunity to let BSCR members know about your research activities and also provide an insight into your research field.

We are keen to hear from anyone in cardiovascular research who would be willing to write for *The* Bulletin.

If you are interested, please contact the Bulletin editors with your ideas: Helen (h.maddock@coventry.ac.uk) or Nicola (N.Smart@ich.ucl.ac.uk)

BSCR Spring Meeting 2003

Molecular Therapy for Cardiovascular Disease

Glasgow 27th-28th March 2003

Organised by Andrew Baker and Sarah George

The field of molecular therapy is a very exciting technology for the treatment of cardiovascular disease. It is a rapidly advancing field and therefore the aim of this meeting was to provide a forum for presentation and discussion of the most up to date information. Furthermore, there is much debate even within the field of the best gene therapy vector and delivery system to use for cardiovascular diseases, so this gathering aimed to provide an oppurtunity for useful discussion. As the cardiovascular gene therapy community is relatively small within the United Kingdom several international speakers were invited to enable a comprehensively programme. The following areas of cardiovascular molecular therapy were discussed:

1. Current gene delivery technology

2. Methods of modifying and improving vectors

3. Cardiovascular disease targets

After registration and lunch **Dr Andrew Baker** welcomed the delegates and wished all an enjoyable and interesting stay in the unseasonably sunny Glasgow. He acknowledged the generous sponsorship of the meeting by the BSCR, British Heart Foundation, The Wellcome Trust, British Cardiac Soceity, National Heart and Lung Foundation, Roche, Invitrogen, Tecan, GRI, Jencons, Applied Biosystems, PAA Laboratories, Medical Air Technology and Power ADInstruments, which allowed this meeting to take place.

The first session entitled 'Current concepts in gene delivery technology' was chaired by **Dr Stuart Nicklin** (Glasgow). The first speaker **Dr Steve Hart** (London) started the session by discussing the potential of non-viral gene delivery vectors. He described the properties of the lipo-peptide, which can be targeted using peptides discovered by phage display. The potential of this approach was illustrated by his studies with transfection of the tissue inhibitor of metalloproteinase-1 (TIMP-1) gene into the adventitia of rat balloon injured arteries which reduced intimal thickening at both 14 and 28 days after injury. The second speaker was **Professor Eric Alton** (London) who very eloquently described his angiogenesis gene therapy studies. Using his experience he highlighted the pros and cons in carrying out such studies when phamaceutical companies are involved. From his findings with the porcine ameroid model he suggested used that multiple genes may be necessary to induce clinically useful neo-angiogenesis. **Dr Chris Newman** (Sheffield) was the third speaker in this session. He described the use of ultrasound to aid gene delivery. This novel technique recently exploited by Dr Newman greatly improves the efficiency of liposome gene delivery in vitro. Furthermore, results are emerging to suggest that this technique has potential for improving the efficiency of liposome gene therapy in vivo. The next speaker **Dr Hildegard Buening** (Munich) changed the focus of the session to a viral method of gene delivery. She described a relatively new vector the adenoviral associated virus (AAV). Although Dr Buening's research focuses on the use of AAV for cancer gene therapy, she illustrated that this vector may also be useful for cardiovascular gene therapy as they have a good safety profile, low immunogenicity and amenability to modification and propagation. The final speaker in this session was **Dr Cathy Holt** (Manchester) who described the use of antisense therapeutics. After describing the principles of this technology, she illustrated why c-myc has been chosen as an antisense target and the success of using this antisense delivered by a gene eluting stent.

We were honoured that **Professor Lawrence Chan** (Baylor Medical College, Texas, USA) gave the British Cardiac Society Lecture entitled ' Adenoviruses and Atherosclerosis: The Potential'. In this excellent lecture Professor Chan described one of the most exciting new gene delivery vectors the helper dependendant adenoviruses. He highlighted that one of the most important advantages of this vector is its ability to produce long-term transgene delivery, however he noted that these vectors are very difficult to make. Using studies where the apolipoprotein E (apoE) gene was re-introduced into apoE deficient mice he illustrated that vectors using genomic DNA produced longer transgene expression than cDNA. Furthermore, atherosclerotic lesions were reduced even at two and a half years after gene introduction. He suggested that the initial studies with apoE, LDL receptor, vLDL receptor and apolipoprotein AI clearly illustrate 'proof-of-principle'. He highlighted that ongoing preclinincal studies aim to improve delivery, assess the ability of one vector to deliver multiple genes, increase the number of helpers and reduce immediate toxicity. He completed his lecture by describing his study aimed at treatment of diabetes mellitus. The study aimed to induce b cell production by gene transfer of neuroD. Initial findings illustrated that neuroD gene transfer partially corrected blood glucose levels and the addition of BTC improved fasting sugar levels further as well as presence of b cell islets. He suggested that this exciting ability to induce islet neogenesis in mice needs more preclinical experiments and may even be useful for ex vivo therapy.

The formal sessions of the first day were then concluded by this inspiring lecture. We then adjourned to a wine reception and poster session. Thirteen excellent posters were displayed on a variety of subjects gave the opportunity for lots of discussion prior to dinner. After the conference dinner we were entertained by our 'after-dinner' speaker **Professor Stewart Hillis** (Glasgow). In addition, to being a cardiologist at the Western Royal Infirmary in Glasgow he is the doctor for the Scottish National Football team. He amused us with his football stories that he illustrated with a slide show.

The focus of the first session of the second day was 'new technology and modified vectors' and was chaired by **Professor Martin Bennett** (Cambridge). **Professor Ketith Channon** (Oxford) as the first speaker discussed the modification of adenoviral vectors. He described how the adenoviral genome has been modified with the aim of reduce immunogenicity and efficiency of these vectors. He illustrated using the rabbit carotid vein graft model that higher persistance, endothelial impairment and inflammation are reduced using E1/E4 deleted vectors compared to E1. He further suggested that targetted vectors and gutless/helper dependent adenovirus may be useful for cardiovascular gene therapy. The next speaker **Dr Eric Biessen** (Leiden) described the use of phage display technology for the identification of targetting ligands. He selected P-selectin as elevated levels are detected in atherosclerosis and using phage display technology he has identified ligands that bind to P-selectin. A consensus sequence was identified in several peptides and it inhibited phage binding to P-selectin as well as HL60 rolling velocity. He illustrated that these peptides may be useful for cardiovascular disease as they targetted thrombus and increased liposome binding. Due to technical difficulties the next speaker was **Dr Len Seymour** (Oxford) who described the use of bFGF virus coating. This increased the resistance to serum knockdown. Another versatile way of modify the vector was polymer coating that also increased resistance, reduces liver transduction and provides systemic biodistribution. The final speaker of the session was **Dr Stuart Nicklin** (Glasgow) who described the use of ligands to enhance the efficiency and generate selectiviey. Ligands identified by bio-panning have been inserted into the fibre of adenoviral and AAV vectors and allowed modification of the tropism.

The session after the morning coffee break chaired by **Dr Chris Jackson** (Bristol) was the first session on disease targets for molecular therapy. **Dr Andrew George** (London) started this session by discussing targetting transplantation. He described the ability of antibodies to target liposome gene delivery and illustrated that anti-E-selectin antibodies increased gene transfer to human synovial vasculature. The next speaker was **Dr Sarah George** (Bristol) who illustrated the potential of TIMP-2 gene transfer for reduction of atherosclerotic plaque rupture. Raising TIMP-2 plasma levels in apoE deficient mice reduced plaque rupture in this model of spontaneous plaque rupture, developed in Bristol by Dr Jackson's group. The next speaker in this session was **Professor Martin Bennett**, he took

a step back and described the multi-step process that have been utilized to identify target genes. He suggested that we need to apply caution to gene array results as they may not be markers of plaque rupture and that we may be focussing on the technology rather than the biology. This session was completed by **Professor Anna Dominiczak** (Glasgow) who described the use of gene transfer for the treatment of hypertension. She suggested that primary hypertension that affects 25% of the adult population requires development of gene transfer despite the availability of drugs due to their side-effects. Her experiments using the stroke-prone sponataneously hypertensive rat have demonstrated that modification of the nitric oxide/superoxide balance with eNOS restores endothelial function and that these effects with AAV and antisense are better than drugs.

The session after lunch comprised of 6 short presentations that were selected from the submitted abstracts was chaired by **Dr Sarah George**. **Dr Tom Johnson** (Bristol) described the use of gene eluting stents to deliver TIMP-3 to porcine coronary arteries, **Miss Laura Denby** (Glasgow) discussed novel adenoviral vectors, **Dr Paul Kingston** (Oxford) described the ability of novel combinations of promoters and enhancers to increase transgene expression, **Dr J Zhao** (Cambridge) discussed the use of lentiviruses to mediate gene transfer of vIL10 to rat cardiac allografts, **Dr Motoki Sato** (London) compared modified direct injection method and intracoronary delivery to cardiac myocytes and Dr Daniel Heaton (Oxford) completed the session by describing NOS-1 gene transfer to promote cardiac vagal neurotransmission and gain of function.

After an afternoon tea break **Dr Keith Channon** (Oxford) presented the poster and oral presentation prizes to **Dr Lorraine Work** and **Dr J Zhao**, respectively. The first speaker in this second session focussing on disease targets was **Professor Andrew Newby** (Bristol). He described the potential of gene transfer for the ex vivo treatment of vein grafts as well as the advantages and disadvantages of animal models of vein graft disease prior to describing anti-proliferative, anti-migratory, pro-apoptotic and anti-inflammatory studies. The next speaker was **Professor George Dickson** (London) who described the ability of second generation adenoviruses to correct hyperlipidemia and the ability of these vectors to provide longer term transgene expression. He also described the more controversial but exciting technique of gene correction.

It was a great pleasure to invite our final speaker **Dr Paul Reynolds** (Adelaide, Australia) to give the National Heart Research Fund Lecture on gene therapy for pulmonary disease. He gave an extremely detailed description of his extensive studies to modify vector technology to aid gene delivery to the pulmonary vasculature. He illustrated cell specific delivery through genetic modification of the vector and prmoters. He suggested that increasing our knowledge of the molecular basis is required to develop new therapy and dual targetting concepts may be useful.

The meeting was closed by **Dr Andrew Baker** who thanked all of the speakers for their excellent presentations and the delegates for contributing to the stimulating discussion that took place in the formal sessions and informally during the meeting.

Submission Deadlines for *The* Bulletin:

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BSCR Spring Meeting 2003: Abstracts

STENT-BASED LOCAL DELIVERY OF THERAPEUTIC ADENOVIRUS EFFECTIVGLY REDUCES NEOINTIMAL PROLIFERATION IN PORCINE CORONARIES.

Thomas Johnson, Yin Xiong Wu, Andreas Baumbach, Andrew Newby, Karl R. Karsch, Martin Oberhoff. Department of Cardiology, Bristol Heart Institute, University of Bristol, Bristol, U.K.

Background: We aimed to assess the effect of locally infecting coronary arteries with an adenovirus (AdV) over-expressing Tissue Inhibitor of Metalloproteinase 3 (TIMP3). TIMP3 plays a crucial role in the regulation of metalloproteinase activity & uniquely promotes apoptosis of smooth muscle cells, thus inhibiting neointimal proliferation.

Method: We used a Matrix HI phosphorylocholine-coated stent (Abbott, USA), modified to elicit increased positive charge, thereby enhancing the binding of negatively charged AdV. Preliminary in-vitro & short-term in-vivo studies were performed to confirm enhanced AdV transduction & transcription with the novel coating. Subsequently, we deployed stents ± TIMP3 AdV, in porcine coronaries for 28 days (n=5 per group). Planimetric measurements & Injury Scores were recorded in 4 sections per stent.

Results: Preliminary work with b-Galactosidase AdV confirmed effective transduction in-vitro. TIMP3 AdV transduction & transcription was demonstrated at 7 days in-vivo, with PCR & Immunohistochemical methods. Comparison with control stents, at 28 days, revealed that TIMP3 stents significantly reduced neointimal area (mm^2) 127.1 ± 84.6 vs 212.1 ± 87.5, $p<0.005$, without any difference in medial area (mm^2) 103.7 ± 21.0 vs 112.6 ± 17.2, $p=0.15$, or injury score (2.0±0.6 vs 1.8 ±0.3, $p=0.36$).

Discussion: Our novel biosynthetic stent coating effectively promotes TIMP3 AdV transduction & transcription, in-vitro & in-vivo. Furthermore, TIMP3 can successfully reduce neointimal proliferation by upto 40% longterm, thus confirming its role in the prevention of in-stent restenosis.

NOVEL ADENOVIRAL VECTORS FOR CARDIOVASCULAR DISEASE.

L Denby, S A Nicklin, D Graham, D J Von Seggern*, A F Dominiczak and A H Baker. Division of Cardiovascular and Medical Sciences,University of Glasgow, Glasgow. *Department of Immunology, The Scripps Institute, La Jolla, CA, USA.

Gene therapy offers a promising new approach to treat cardiovascular disease. Systemic delivery of the gene delivery agent would be highly desirable for many applications. At present this goal has proven elusive. The major limiting factor is the highly efficient sequestration of virions, such as those based on recombinant adenovirus (Ad) type 5-based vectors to the liver. Therefore, it is important to identify novel adenoviral vectors that have a more favourable infectivity profile. Pseudotyping is a technique by which the Ad5 fiber can be exchanged for any of the 50 other fibers of alternate serotypes. Using this technology we have sought to evalute the infectivity of candidate fibers from Subgroup B, C and D for vascular cells *in vitro* and *in vivo*. Subgroup D viruses had a markedly different infectivity profile when compared to Ad5. Subgroup D vectors transduced human smooth muscle cells (SMC) and endothelial cells (EC) at levels equivalent to, and or higher than Ad5 (EC 60.6% ± 19% vs Ad5; SMC 248%±42% vs Ad5). However in stark contrast to Ad5 the Subgroup D fibers did not support transduction of human hepatocytes (0.22%±0.06% vs Ad5). Similar results were obtained in rat cells *in vitro*. Importantly, when we sytemically injected the subgroup D vectors into WKY rats we observed a 45% reduction in virion accumulation in the livers compared to Ad5 assessed by realtime PCR ($p<0.05$) at 1 hour. At 5 days post infusion ony Ad5 DNA could be detected in the liver. X-gal staining of the Subgroup D vector livers showed a remarkable reduction in liver transduction at 5 days compared to Ad5. These findings indicate that a Subgroup D system will be useful for the development of systemically-injectable, vascular-targeted gene therapies.

ADJUNCTIVE ULTRASOUND EXPOSURE (USE) ENHANCES TRANSGENE EXPRESSION FOLLOWING TRANSFECTION OF PORCINE VASCULAR SMOOTH MUSCLE CELLS (VSMC) AND SAPHENOUS VEIN (SV).

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Background: We have shown that (USE) in the presence of the albumin shell microbubble echocontrast agent (MECA) Optison™ enhances transgene expression in VSMC following naked DNA transfection by up to 300-fold. This study investigated the effects of USE on transfection of intact vessels and isolated VSMC using a new generation of phospholipid (PL) shell MECA.

Methods: VSMC were transfected with *Lac Z* or luciferase (*luc*) plasmid ± USE at 1MHz for 60s in the presence of the PL MECA BR14 (Bracco). SV segments were transfected with *luc* plasmid ± 100mmHg non-distending static pressure for 5 min ± subsequent USE. Samples were analysed for *luc* / *Lac Z* expression at 48h (VSMC) or 72h (intact SV) by luminometry or X-gal staining.

Results: USE with BR14 enhanced *luc* expression in VSMC by 3000-fold compared to DNA alone ($1.2\pm0.18 \times 10^6$ *vs* $0.4\pm0.05 \times 10^3$ light units (LU)/mg cell protein; $p<0.001$, n=4); the number of *Lac-Z* positive cells was also markedly enhanced (4.7±0.9 *vs* 0.1±0.1%; p<001, n=3). Static pressure alone did not increase *luc* expression in intact SV compared with DNA alone (2.6 ± 3 vs $2.3\pm4 \times 10^4$ LU/g tissue weight). In contrast, the enhanced *luc* expression after USE alone was further increased using the combination of static pressure followed by USE (from 44.7 ± 14 to $147 \pm36 \times 10^4$ LU/g; $p<0.05$ for these comparisons, n=6).

Conclusion: USE in the presence of BR14 MECA markedly improves plasmid transfection of intact SV and isolated VSMC. These data give encouragement to further studies *in vivo.*

COMPARISON BETWEEN A MODIFIED DIRECT INJECTION METHOD AND INTRACORONARY DELIVERY FOR *IN VIVO* GENE TRANSFER TO CARDIAC MYOCYTES.

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We already reported a modification to the direct injection (DI) method (Immobilisation technique; i-DI) that can markedly improve transfection efficiency. The aim of this study was to compare i-DI with intracoronary injection (IC) in terms of transfection efficiency and efficacy of transgene product. Sprague-Dawley rats were injected with E1-deficient-type adenovirus, having phospholamban-antisense cDNA plus green fluorescent protein (Ad.PLB-as.GFP, 10^9 pfu in 100µl for DI and 10^{10} pfu in 200µl for IC). For DI and IC, the rats were killed and myocytes isolated two to five days and seven days after injection respectively. Sufficient transfected myocytes for contraction experiments (>1% approximately) were obtained in 7 of 7 i-DI infections compared with 6/16 IC. Myocytes transfected by either i-DI and IC showed evidence for functional effects of PLB down-regulation, with a significant increase in % shortening at 8mM Ca^{2+} from 9.55 ± 1.27 to 13.1 ± 0.9 ($p<0.05$) and 8.49 ± 1.51 to 13.6 ± 1.5 ($p<0.05$) respectively. We conclude that Immobilized-direct injection in our hands showed a better transfection efficiency than intracoronary injection. Since the amount of vector used was 20-fold less for i-DI, this method has advantages for experimental investigations requiring *in vivo* gene transfer to myocytes.

A NOVEL COMBINATION OF PROMOTER AND ENHANCERS INCREASES TRANSGENE EXPRESSION IN SMOOTH MUSCLE CELLS AND IN CORONARY ARTERIES AFTER ADENOVIRUS-MEDIATED GENE TRANSFER.

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The vascular smooth muscle cell (SMC) is the target for gene transfer in a variety of "therapeutic" settings within the vasculature. However, SMC are not a good target for gene transfer. Powerful viral promoters (e.g. the major immediate-early murine cytomegalovirus enhancer/promoter - MIEhCMV) remain largely silent from a transcriptional perspective within SMC. We have previously demonstrated that the major immediate-early murine cytomegalovirus enhancer/promoter (MIEmCMV) elicits greater transgene expression in SMC than MIEhCMV. Using a selection of adenoviruses containing *lacZ*, we have investigated the ability of other enhancers of transgene expression (the Woodchuck hepatitis virus post transcriptional regulatory element - WPRE, and a fragment of the rabbit smooth muscle myosin heavy chain promoter - RE) to further increase transgene expression in SMC *in vitro* and in pig coronary arteries after Infiltrator catheter-mediated gene transfer. At MOI=10, an adenovirus containing MIEmCMV, WPRE and RE elicited 90-fold greater b-galactosidase expression in SMC *in vitro* than a vector containing MIEhCMV alone ($p<0.001$), and ~4-fold greater expression than vectors containing MIEmCMV alone and MIEmCMV+WPRE (both $p<0.001$). Within pig coronary arteries, $2x10^9$iu of vector containing MIEmCMV, WPRE and RE gave rise to 20- to 40-fold greater expression of β-galactosidase in lysates of infected vessel segments than $2x10^9$iu of those vectors containing MIEhCMV alone, MIEmCMV alone or MIEmCMV+WPRE (all $p<0.05$). The combination of MIEmCMV, WPRE and RE offers the potential for substantially greater levels of transgene expression than it has previously been possible to achieve after gene transfer into SMC and the vasculature.

SITE-SPECIFIC *IN VITRO* GENE DELIVERY TO VASCULAR TISSUE USING PEPTIDE-TARGETED VIRAL VECTORS.

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The endothelium is intrinsically involved in numerous vascular pathologies; hence we have sought to generate vectors specifically targeted to endothelial cells (EC). Using a random 12 amino acid peptide phage display library, we isolated peptides capable of directing binding to primary human EC. We choose two of these peptides based on frequency of isolation, target cell binding analysis and the ability to also bind murine EC lines. Each peptide [MSLTTPPAVARP (MSL) and MTPFPTSNEANL (MTP)] was inserted into adenoviral (Ad) and adeno-associated viral (AAV) vectors. In vitro analysis of retargeted Ad and AAV demonstrated selectivity for EC, with competition studies demonstrating that EC binding was independent of native tropism. Tropism *in vivo* following intravenous injection into 5-week old male Balb-C mice was evaluated by quantification of vector DNA in organs 1 hour post-administration using real-time PCR. Gene expression at 5 days for Ad and 28 days for AAV was also determined. Retargeted AAV vectors showed a decrease in liver accumulation and gene expression with a longer circulating half-life, also demonstrated an increase in accumulation and transduction of venous tissue. These results constitute a significant advance in viral vector design.

ENGINEERING VIRAL VECTORS WITH SMOOTH MUSCLE CELL TARGETING PEPTIDES.

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Local delivery of gene therapy vectors to the vessel wall holds potential for the treatment of late vein graft failure and restenosis. To identify novel targeting peptides that selectively bind human saphenous vein smooth muscle cells (SMC) we performed *in vitro* biopanning on SMC. Phage were incubated with SMC in order to isolate peptides which could selectively improve adenovirus (Ad) or adeno-associated virus (AAV) transduction of SMC. Analysis of 96 encoded peptide inserts identified 9 novel consensus motifs and 2 consensus peptide sequences, these consensus peptides (GLA1 and GLA2) were incorporated into the HI loop of Ad. Short exposures (<1 hr) of the modified virus to SMC significantly improved reporter gene expression in the absence of endothelial cell transduction for GLA1 but not GLA2. In addition we incorporated GLA1 into the VP3 capsid protein of AAV. A significant enhancement in reporter gene expression with the modified AAV vs non-modified control was observed at short exposure times. Identification of SMC targeting peptides by phage display and their incorporation into viral vectors has important implications for the treatment of late vein graft failure and post-angioplasty restenosis enabling selective and efficient gene delivery to SMC.

IDENTIFICATION AND EVALUATION OF PLAQUE TARGETING PEPTIDES BY PHAGE DISPLAY.

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We have utilized two phage display approaches to identify short peptide ligands which could support specific binding of gene therapy vectors to atherosclerotic plaques. In the first, *in vivo* phage biopanning was performed against the braciocephalic artery in 3-month hi-fat fed apoE$^{-/-}$ mice, which represents a consistent and reproducible site of plaque formation in these animals. Recovery of phage from the braciocephalic artery *in vivo* increased between 2 and 4 logs over four rounds of duplicate panning using a 12mer peptide library with an initial input titer of $4x10^{12}$ pfu phage which was decreased to $5x10^{9}$ pfu and $1x10^{9}$ pfu over the following rounds. 110 phage were sequenced from the third round revealing 79 unique sequences and eight peptides bearing identical motifs of at least 4 amino acids. To supplement the *in vivo* biopanning, *in vitro* panning was performed against microtiter wells coated with 1.5 mg vascular cell adhesion molecule 1 (VCAM-1) using both 12mer and cyclic 7mer libraries in triplicate with a fixed input titer of $4x10^{10}$ pfu. An increased recovery of phage of between 1 and 3 logs was observed over three rounds for both libraries, following which individual phage were screened for their ability to selectively bind to VCAM-1 by an ELISA-based assay. Phage selected from both the *in vivo* panning and those with affinity for VCAM-1 were screened for their ability to bind to TNF-stimulated primary human endothelial cells *in vitro*. Several individual phage isolated by each of these methods showed up to five-fold higher binding to stimulated endothelial cells over quiescent cells *in vitro*, and also demonstrated reduced binding of non-endothelial cell types indicating selectivity for endothelial cell receptors.

ENHANCED CARDIAC VAGAL PHENOTYPE FOLLOWING EXERCISE-TRAINING IS ABSENT IN MICE LACKING ONE NEURONAL NO SYNTHASE ALLELE.

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We investigated the role of neuronal NO-synthase (NOS-1), present in parasympathetic neurones, in facilitating increased vagal responsiveness which is enhanced following exercise training. Male wild-type (WT,+EX; n=12; 9.5±0.8 km/day) and heterozygous NOS-1 knockout (NOS-1+/-,+EX; n=12; 9.1±1.8 km/day) underwent 5 weeks voluntary wheel-running and were compared to control mice without wheels (WT, -EX n=12; NOS-1+/-, -EX n=12). In WT, +EX atria, HR responses to vagal nerve stimulation (VNS) *in-vitro* were significantly enhanced compared to WT,-EX atria ($P<0.05$); but responses were unaffected in NOS-1+/-,+EX compared to NOS-1+/-,-EX . Inhibition of NOS-1 with L-VNIO attenuated HR responses to VNS in all atria ($P<0.05$) and normalized the responses in WT, +EX with respect to WT,-EX atria. Effects of L-VNIO on VNS responses were reversed by L-arginine. Western Blot analysis confirmed that expression of NOS-1 protein in atria was increased in WT, +EX compared to WT,-EX (by 78%; $P<0.05$). Basal expression of NOS-1 in NOS-1+/-,-EX was not different compared to WT,-EX atria and NOS-1+/-, +EX was not increased with respect to NOS-1+/-,-EX. In-vivo cardiac gene transfer using adenoviruses containing NOS-1 increased vagal bradycardia in isolated NOS-1+/-, +EX atria compared to gene transfer of eGFP ($P<0.01$). This difference was abolished by NOS-1 inhibition using L-VNIO. In conclusion, exercise training enhances the HR response to peripheral vagal nerve stimulation by a NO-dependent mechanism. These training-induced changes are dependent on two intact NOS-1 alleles, although the impaired phenotype can be rescued by cardiac NOS-1 gene transfer.

ENHANCEMENT OF LIPOPOLYSACCHARIDE-STIMULATED JNK ACTIVITY IN RAT AORTIC SMOOTH MUSCLE CELLS BY PHARMACOLOGICAL AND ADENOVIRUS-MEDIATED INHIBITION OF INHIBITORY KAPPA B KINASE SIGNALING.

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In rat aortic smooth muscle cells, the putative NFkB inhibitor Pyrrolidine dithiocarbamate (PDTC) was found to inhibit lipopolysaccharide (LPS)-stimulated NFkB DNA-binding. However , the site of inhibition was found to be at, or upstream of, the inhibitory kappa B kinases (IKKs). PDTC also potentiated LPS-stimulated JNK, p38 MAP kinase and MAPKAP kinase-2 activity. Another inhibitor of NFkB signalling, the serine protease inhibitor Nα *p*-Tosyl-L-lysine chloro-methylketone also inhibited LPS-stimulated IKK activity and potentiated JNK activity in response to LPS, suggesting that cross-talk may occur between the NFkB and SAP kinase pathways at the level of IKK or at a common point upstream. Infection with an adenovirus encoding either a dominant-negative IKKβ or inhibitory kappa B-α (which is downstream of IKK) also potentiated LPS-stimulated JNK activity, indicating that the loss of NFkB DNA-binding, rather than the loss of IKK activity, is sufficient to cause the increase in JNK activity. This shows that inhibition of NFkB DNA-binding enhances JNK activation in vascular smooth muscle cells, an effect that may contribute to the pathophysiological effects of LPS.

NOS-1 GENE TRANSFER PROMOTES CARDIAC VAGAL NEUROTRANSMISSION AND GAIN OF FUNCTION.

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We tested the hypothesis that NOS-1 gene transfer to the right atrium would enhance vagal bradycardia, since pharmacological evidence implicates the NO-cGMP pathway in facilitation of cholinergic neurotransmission. Percutaneous gene transfer was performed in male guinea pigs, using adenoviruses encoding NOS-1 (Ad.NOS-1) or eGFP (Ad.eGFP). Some animals received a sham injection of saline alone. After ~5 days incubation, NADPH-diaphorase staining and Western blotting showed increased right atrial NOS-1 expression in the Ad.NOS-1 group relative to the control groups. Confocal imaging showed co-expression of NOS-1 with ChAT, indicating increased expression in cholinergic ganglia. Heart rate responses to 3 and 5Hz right vagal nerve stimulation were increased in the NOS-1 group, both *in vivo* and *in vitro*. This was associated with increased release of [^{3}H]ACh in response to 10Hz field stimulation. *In vitro* application of the NOS-1 inhibitor Nω-nitro-L-arginine attenuated the enhanced vagal bradycardia in the Ad.NOS-1 group, while ODQ normalised release of [^{3}H]ACh. This suggests that NOS-1 acts pre-synaptically to enhance vagal bradycardia, and that up-regulation of NOS-1 via gene transfer may provide a novel method for inducing cardiac vagal gain of function. ***Circ Res.* 2002;91: 1089-1091.**

CELL-CELL CONTACT BY CADHERINS PROVIDES AN ESSENTIAL SURVIVAL SIGNALTO MIGRATING VASCULAR SMOOTH MUSCLE CELLS.

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We examined the effect of disrupting cadherins on VSMC migration. Human saphenous vein VSMCs were grown to confluence and then wounded. Migration and the percentage of apoptotic VSMCs by in situ end labelling were assessed after 24 hours. Selective inhibition of cadherin function using 200mg/ml cadherin binding site inhibitory peptide (HAV) significantly reduced migration by 53±8% compared to the control peptide (n=3, Student t-test, $p<0.05$). Furthermore, inhibition of only N-cadherin function using 80mg/ml neutralizing antibodies and adenoviral expression of dominant negative N-cadherin significantly reduced migration by 33±1% and 40±12% compared to non-immune immunoglobulin and reporter gene controls, respectively (n= 3, $p<0.05$). Death of VSMC was significantly increased by inhibition of cadherin function (HAV peptide 3±1-fold, N-cadherin antibody 3±1-fold, dominant negative N-cadherin 4±1 fold, n=4, $p<0.05$). This increase in cell death clearly contributes to reduced migration of VSMCs. This study indicates that cell-cell adhesion mediated by cadherins, particularly N-cadherin, is a survival signal for migrating VSMCs. We suggest disruption of cadherins is a potential strategy for reducing VSMC migration and intimal thickening.

OVEREXPRESSION OF INTERLEUKIN-18 DECREASES PLAQUE STABILITY IN FEMALE APOLIPOPROTEIN-E KNOCKOUT MICE.

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In apoE-/- mice IL-18 enhances atherogenesis through release of IFNγ. Male IFNγ -/- x apoE-/- mice showed decreased lesion size whereas in females IFNγ deficiency had no such effect. This study aims to assess the effect of IL-18 on plaque stability in female apoE-/- mice. We induced atherosclerosis in the carotid arteries by perivascular collar placement. To evoke a systemic up-regulation of IL-18 we injected an adenoviral vector intravenously. Two weeks later the carotid arteries were harvested. Plaques were measured, classified and scored for adverse events. We found no difference in plaque size in the IL-18 treatment group compared to the control animals. The ratio of macrophage to plaque area was slightly decreased in the IL-18 group ($p=0.045$). Furthermore in this group we found 10/16 vessels with morphology prone to rupture or even rupture or haemorrhage as compared to 4/17 in the controls. The IL-18 treated mice showed decrease in collagen/intima ratio ($p=0.003$). This suggests that systemic IL-18 overexpression causes a decrease in collagen content and leads to a more vulnerable phenotype in female apoE-/- mice.

ENHANCED NON-VIRAL VASCULAR GENE DELIVERY USING VP22-MEDIATED INTERCELLULAR PROTEIN TRANSPORT.

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Background Fusion of the Herpes Simplex Virus (HSV) gene VP22 to a number of plausibly therapeutic transgenes results in intercellular trafficking of the fusion protein from a single transfected cell to up to 200 surrounding cells, following adenoviral transfection. The effectiveness of plasmids encoding fusions between VP22 and two genes with potential relevance to cardiovascular disease, HSV-I Thymidine kinase (TK) and the dominant negative c-myb allele, MybEngrailed (MybEn), have been investigated, using non-viral transfection of vascular smooth muscle cells (VSMC) *in vitro*.

Methods and Results Plasmids encoding TK, MybEn and the relevant VP22 fusions were transiently transfected into porcine VSMC using polyplex transfection. All the VP22 fusions tested exhibited intercellular trafficking in VSMC. TK transfection was associated with substantial (³ 70%) cell death in the presence of ganciclovir despite a low (<2%) primary transfection rate, indicating a substantial bystander effect in these cells. Consequently, transfection with VP22-TK fusions was not associated with additional cytotoxicity. In contrast, transfection with MybEn-VP22 was associated with a 10.6 ±3.6 fold greater inhibition of VSMC population growth and a 3.2 ±1.0 fold increase in apoptosis compared with transfection with MybEn alone.

Conclusion Plasmids encoding fusion proteins of VP22 and therapeutic transgenes, which exhibit highly efficient intercellular trafficking following transfection, may enhance the effectiveness of non-viral vascular gene delivery.

SYSTEMIC ADMINISTRATION OF ADENOVIRUS EXPRESSING POTENTIALLY THERAPEUTIC GENES IN A RAT MODEL OF HYPERTENSION.

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We have previously shown that adenoviral-mediated gene transfer of extracellular superoxide dismutase (SOD3) or endothelial nitric oxide synthase (eNOS) improves endothelial function in the stroke-prone spontaneously hypertensive rat (SHRSP). The aim of the present study was to deliver these genes systemically and determine the effect on SHRSP blood pressure. Systolic blood pressure (SBP) was continuously recorded by radiotelemetry in male, 11-week old SHRSPs for a total of 8 weeks. After 5 days' baseline recording, rats received a pre-dose of empty virus. Ad vectors, or vehicle, encoding for eNOS, SOD3 or EGFP were infused via the femoral vein 4 hours later. Additionally, one group received AdECSODDHBD, encoding for a heparin-binding domain deleted ecSOD that may be effective at areas distant to the site of expression. Biodistribution of infused virus using real time PCR revealed the majority of adenoviral particles to be accumulated in liver and spleen. Immunohistochemistry showed that transgene expression paralleled this. However, no significant change in SBP occurred between the animal groups at any time point. AdECSODDHBD also failed to affect SBP. Refinement of adenoviral-mediated gene delivery to the vasculature is required in order to pursue this method as a systemically injectable intervention against hypertension.

A GENE TRANSFER STRATEGY TO ALTER THE PHENOTYPE OF CALCIFYING VASCULAR CELLS BY BLOCKING DIFFERENTIATION INTO CALCIFIED NODULES *IN VITRO*.

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The common finding of calcification in vascular medicine underpins the importance for understanding its pathogenesis. Proteins known to be involved in the controlled calcification that occurs during osteogenesis, such as BMP-2 & 6, osteopontin and MGP are also found in calcified atherosclerotic lesions. This knowledge provides the impetus to analyse and characterise the genes involved in the pathogenesis of calcification, as this could lead to the development of novel targets for therapeutic intervention. To this end, subtractive hybridisation and RLM-RACE has been used to identify and clone a novel gene, designated clone 15, which is differentially expressed in an *in vitro* model of calcification. The predicted amino acid sequence of this novel gene encodes a protein of 15KDa, similar in size to many growth and differentiation factors. Northern blot analysis shows a transcript of 800bp, which is up-regulated when vascular cells deposit a mineralised matrix. We show expression of clone 15 in human calcified atherosclerotic lesions using RT-PCR. We propose that the protein encoded by this novel cDNA plays a key role in regulating the deposition of a calcified matrix both *in vitro* and *in vivo.* Functional analysis of clone 15 is being performed using an adenoviral-mediated over-expression strategy during vascular cell differentiation. Current studies are testing the hypothesis that up-regulation of clone 15 is sufficient to cause calcification of vascular cells, and that interfering with expression of clone 15 in a molecular manner *in vitro* with an antisense cDNA construct will prevent mineralization.

LENTIVIRUS-MEDIATED GENE TRANSFER OF VIRAL INTERLEUKIN 10 PROLONGS SURVIVAL OF CARDIAC ALLOGRAFTS IN RATS.

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Introduction: Lentiviral vectors are emerging as the vectors of choice for *in vitro* and *in vivo* gene therapy studies due to their long term gene expression and the lack of host immune response. In this study we examined the feasibility of transferring the viral interleukin -10 (vIL-10) gene into rat cardiac grafts using lentiviral vectors by direct intramyocardial injection.

Methods: Lentiviral vectors containing vIL-10 were generated from an HIV-1 construct, in which the viral promoter had been inactivated and virtually all the viral accessory proteins had been deleted in order to give the maximum safety. vIL-10 gene expression in transduced hearts was examined by RT-PCR. Cardiac allograft transplants were performed in highly histocompatible rat strains (Lewis to DA).

Results: Long term vIL-10 expression was shown in the heart isografts at least 28 days after transduction. Animals transduced by vIL-10 showed significantly prolonged allograft survival without conventional immunosuppression (14.5 ± 1.0 days vs. 8.0 ± 0.7 days for control, $p < 0.001$).

Conclusion: Expressing vIL-10 in rat heart using lentiviral vectors prolongs allograft survival. The survival time is comparable to that using adenoviral vectors delivering vIL-10 in a similar rat strains combination, suggesting that the limited survival may be due to lack of inhibition of early phase of alloimmune response.

FUNCTIONAL OXYTOCIN RECEPTORS ON PRIMARY HUMAN VASCULAR SMOOTH MUSCLE CELLS.

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Vascular smooth muscle cells (VSMC) in healthy vessels are primarily in a contractile phenotype. In atherosclerosis contractile cells convert to a secretory phenotype in response to mitogenic signals and migrate in to plaques at sites of disease. In this study we measured the cell surface expression of oxytocin receptors (OTRs) on primary human umbilical (Um) and aortic (Ao) VSMCs in the contractile and secretory phenotypes and investigated whether an oxytocin receptor agonist had mitogenic functions by conferring a proliferative, secretory phenotype on contractile cells. VSMC were grown in 12 well plates. Cells were converted from secretory to a contractile phenotype by withdrawal of growth factors and culture in the presence of low serum (0.25% v/v). Expression of OTRs was measured using whole cell ligand binding. For proliferation studies contractile cells were treated with TGOT, a selective oxytocin agonist, for 24 hours and cell proliferation quantified. The cell surface expression of OTRs (fmol.mg protein^{-1}) were as follows: Um-secretory, 15±2 (n=9); Um-contractile, 36±5 (n=9); Ao-secretory, 17±4 (n=7); Ao-contactile, 94±7 (n=7). TGOT induced proliferative responses in both the Um and Ao contractile VSMC. In conclusion: We found that OTRs were expressed on both Um-VSMC and Ao-VSMC. OTRs were expressed at greater density on VSMC in a contractile phenotype. OTRs are functionally coupled to a proliferative response in contractile VSMC.

This work was supported by the British Heart Foundation grant P/G 99053.

INHIBITION OF MATRIX METALLOPROTEINASE ACTIVITY INCREASES ATHEROSCLEROTIC PLAQUE SIZE IN A MOUSE MODEL OF PLAQUE RUPTURE.

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We hypothesised that administration of a broad-spectrum matrix metalloproteinase (MMP) inhibitor (RS-130830) would both stabilise and limit the progression of brachiocephalic artery atherosclerosis in the apolipoprotein E knockout mouse (apoE$^{-/-}$) model of plaque rupture. Two groups of 24 male mice aged six to eight weeks were placed on a high-fat Western diet. One group had their diet supplemented with RS-130830. Eight weeks later the mice were terminated and the brachiocephalic artery removed. Treated animals had a mean plasma concentration of RS-130830 of 311±45nM (mean±SEM), 50-fold higher than the IC50 for most MMPs. This resulted in a two-fold increase in atherosclerotic lesion size compared with controls (0.074±0.009μm^2 v 0.037±0.006μm^2; p=0.005). There was no significant effect on either the number of healed plaque ruptures, as evidenced by buried fibrous layers within the plaque, or the number of acute plaque ruptures. The tunica media in animals treated with the MMP inhibitor had an increased collagen content (23±2% v 15+2%; p=0.01) and a decreased elastin content (53±2% v 59±2%; p=0.04), with a marked increase in medial area (0.078±0.002μm^2 v 0.066±0.003μm^2; p=0.003). These results demonstrate that administration of a broad spectrum MMP inhibitor at early time points in an animal model of plaque rupture neither suppresses atherosclerotic plaque development nor alters the stability of the lesions. Although these findings may not support the use of broad spectrum MMP inhibitors in the treatment of unstable plaques, additional data from later time points, including survival rates, are needed.

Cardiovascular Related Meetings

Scientific Sessions of the American Heart Association. Orlando, Florida. Enquiries: American Heart Association, Meetings and Councils, 7272 Greenville Avenue, Dallas, TX 75231. Tel.: + 1 214 706 1543; Fax: +1 214 373 3406; E-mail: gcordis@neuron.uchc.edu; Website: http://ishr2003.uchc.edu

XVIII World Congress of the International Society for Heart Research, August 7-11, 2004, Brisbane, Australia. Enquiries: ISHR 2004 Congress, PO Box 164, Fortitude Valley QLD 4006, Australia. Tel +61 7 3854 1611; Fax +61 7 3854 1507; E-mail: heart2004@ozaccom.com.au; Website: www.baker.edu.au/ISHR

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February 2003 to May 2003

Senior Research Fellowships In Clinical Science

Dr S Bhattacharya, Department Of Cardiovascular Medicine, John Radcliffe Hospital, University Of Oxford. Genetic And Environmental Mechanisms In Congenital Heart Disease. 60 Months, £1,491,905

Collaborative Research Initiative Grants

Professor Homero Rubbo, Department Of Medical Biochemistry, University Of Wales College Of Medicine, Cardiff Wales. Biological Properties Of Arachidonate-Derived Nitrated Lipids. 36 Months, £112,675.

Equipment

Dr R M Tribe, Department Of Women's Health, Maternal And Fetal Research Unit, Guys, King's And St Thomas' School Of Medicine,Lambeth Palace Road. Real-Time Pcr (Abi Prism 7000): A Multiuser Facility For Reproductive Research. 36 Months, £20,157.

Entry Level Fellowships For Medical And Dental Graduates

Dr Kelvin Lee, Department Of Cardiology, , University Of Newcastle,Newcastle Upon Tyne. A Genomic Approach To Atherosclerotic Plaque Vunerability. 12 Months, £60,136.

Mr Neil Cartwright, Royal Brompton And Harefield Nhs Trust,London. Mechanism Of Vascular Dysfunction In Human Sepsis: Implications For Improved Therapies. 12 Months, £53,730.

Project Grants

Professor Robert C Read, Division Of Genomic Medicine, Section Of Functional Genomics, University Of Sheffield. Role Of Bacterial Denitrification In The Pathogenesis Of Sepsis. 36 Months, £215,290.

Dr Phillip Eaton, Centre For Cardiovascular Biology And Medicine, Rayne Institute, King's College London. Redox Signalling And Ischaemic Preconditioning: A Potential Role For Protein S-Thiolation. 36 Months, £175,699.

Dr Adrian J Hobbs, Wolfson Institute For Biomedical Research, University College London. Characterisation Of C-Type Natriuretic Peptide (Cnp) As An Endothelium-Derivedhyperpolarising Factor: Regulation Of Vascular Tone, And Platelet Reactivity. 36 Months, £130,460.

Dr Andrea E Munsterberg, School Of Biological Sciences, University Of East Anglia,Norwich. Regulation Of Mesodermal Cell Movement And Fate By Wnt Signalling Pathways In Chick Embryos. 36 Months, £185,683.

Dr David R Poyner, Pharmaceutical Science Research Institute, Aston University, Birmingham. Characterisation Of Conserved Residues And Activation Mechanisms Of The Cgrp Receptor (Cl/Ramp1); A Comparison With The V1a Vasopressin Receptor. 36 Months, £152,703.

BSCR Autumn Meeting 2003

Oxidative Stress: from Measurement to Management

held jointly with the Scottish Cardiovascular Forum

Dates: 8th and 9th September, 2003

Venue: John MacIntyre Conference Centre, University of Edinburgh

Organisers: Gillian Gray, Ian Megson, Simon Maxwell, Irfan Rahman & Ajay Shah

Overall Aims:

- To consider the sources of oxidative stress and cellular targets in cardiovascular disease.
- To discuss the pros and cons of the techniques used for measurement of oxidative stress.
- To examine different approaches to the management of oxidative stress.

Invited Speakers include: Barry Halliwell (*Singapore*), Rudolph Riemersma *(Edin)*, Ingrid Fleming (*Frankfurt*), Sandy Hill (*Dundee*), Jurg Muller (*Magnatech*), Valerie O'Donnell (*Cardiff*), Keith Channon (*Oxford*), Roberto Motterlini (Imperial) Irfan Rahman (*Edin*), Philip Eaton *(KCL)*, Mary Cotter (*Aberdeen*), Ajay Shah (*KCL*), Ralf Brandes (*Frankfurt*), Lucilla Poston (GKT), Julia Brosnan (*Glasgow*), Justine Davies (*Dundee*), Dipak Das (Connecticut), Jane Armitage (*Oxford CTSU*).

Travel & Accommodation: The conference centre is located adjacent to Holyrood Park and close to Edinburgh city centre. En-suite rooms and parking are available on site.

Communications: Part of this meeting will be devoted to oral presentation of selected abstracts, and posters. Prizes will be given for the best oral and best poster presentation given by young investigators.

Registration: Free to BSCR members, £40 for non-members.

Bursaries: The Society will consider awarding travel grants of up to £150 to bona fide PhD students.

Deadline for submission of abstracts, registration and application for student bursaries: 1st August

The abstract pro-forma, meeting registration form, and forms for application for BSCR membership or student bursaries can be downloaded from: http://www.bcs.com/affiliates/bscr.html

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