TOPICAL REVIEW

'Integrative Physiology 2.0': integration of systems biology into physiology and its application to cardiovascular homeostasis

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Abstract Since the completion of the Human Genome Project and the advent of the large scaled unbiased '-omics' techniques, the field of systems biology has emerged. Systems biology aims to move away from the traditional reductionist molecular approach, which focused on understanding the role of single genes or proteins, towards a more holistic approach by studying networks and interactions between individual components of networks. From a conceptual standpoint, systems biology elicits a 'back to the future' experience for any integrative physiologist. However, many of the new techniques and modalities employed by systems biologists yield tremendous potential for integrative physiologists to expand their tool arsenal to (quantitatively) study complex biological processes, such as cardiac remodelling and heart failure, in a truly holistic fashion. We therefore advocate that systems biology should not become/stay a separate discipline with '-omics' as its playing field, but should be integrated into physiology to create 'Integrative Physiology 2.0'.

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Introduction

Maintenance of homeostasis is essential for survival of an organism. The cardiovascular system has therefore developed a high degree of plasticity to maintain circulatory homeostasis in a wide variety of circumstances. Defence mechanisms include acute adjustments, e.g. the cardiovascular adaptations to a sudden increase in physical activity, as well as chronic adjustments, e.g. cardiac remodelling to a chronic elevation in haemodynamic loading conditions following myocardial injury, volume or pressure overload. These adjustments require highly integrated and orchestrated responses involving a large number of controlled variables. In view of the importance of adequate circulatory responses for the survival of an organism, these processes are characterized by a high level of redundancy involving complex signalling pathways that display significant interactions at multiple levels. Integrative physiology has been able to decipher many aspects of cardiovascular homeostasis, including the regulation of coronary blood flow (Duncker & Bache, 2008) as well as the short- and long-term regulation of

Dirk J. Duncker, **Jolanda van der Velden**, **Diederik Kuster**, **Daphne Merkus** and **Adrie Verhoeven** work in the Cardiovascular Research Institutes of Rotterdam and Amsterdam and collaborate on research into the pathogenesis and therapies of cardiac remodelling and dysfunction following acute myocardial infarction. Their backgrounds are in biochemistry (D.K., J.vdV., A.V.), molecular biology (D.K., A.V.) and physiology (D.M., J.vdV., D.J.D.). Starting from *in vivo* observations in exercising pigs, they employ an integrative approach to unravel the cellular, biochemical and molecular basis of cardiac remodelling and dysfunction.

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blood pressure and cardiac function (Guyton, 1992; Hester *et al*. 2011). In other areas of cardiovascular homeostasis, including cardiac hypertrophy, integrative physiology has provided tremendous insight into this process at the organ and cellular level, but only very limited insight into its molecular basis (Fig. 1). The emergence of the field of molecular biology has enabled cardiovascular researchers to obtain deeper insight into this complex process (Mudd & Kass, 2008).

Initial molecular studies in the cardiovascular field principally consisted of observational work, looking at gene and/or protein expression and changes therein in cardiovascular disease states (e.g. Katz, 1988; Brand *et al.* 1992). These studies were followed by more mechanistic approaches to test the involvement of identified (novel) genes and their products, mainly by virtue of knocking out and/or over-expressing a gene of interest (Frey & Olson, 2003; Heineke & Molkentin, 2006). This reductionist approach has significant value in monogenic diseases. However, the use of genetic models in studies of cardiovascular disease soon illustrated the complexity of cardiovascular diseases, as many gene knock-out animal models lacked a clear phenotype. These findings were initially interpreted to suggest that the gene was not important, while a more physiological interpretation is that other genes increased their activity and acted to compensate. These observations, in conjunction with the completion of the Human Genome Project and the advent of the '-omics' technologies, stimulated the emergence of the field of systems biology. As outlined elsewhere in this issue of *The Journal of Physiology*, systems biology aims to move beyond the traditional reductionist molecular approach (which focused on understanding the role of single genes or proteins), towards a more holistic approach by studying networks and interactions between individual components of networks. The strength of this integrative molecular approach is that, even when a perturbation in a molecular pathway does not result in clear phenotypic changes, the responsible compensatory adaptations will likely be mirrored in adaptations in the transcriptome, proteome and/or metabolome. Until now, systems biology has been mainly considered a research field in its own right. However, to date systems biology has been applied to relatively simple systems, including cultured cells and bacteria, but has not been applied to studies of homeostasis in complex organisms, including mammals, a field that has traditionally been the domain of integrative physiology (Fig. 1). We believe that integration of the complementary disciplines of systems biology and integrative physiology is essential to advance our understanding of complex biological processes.

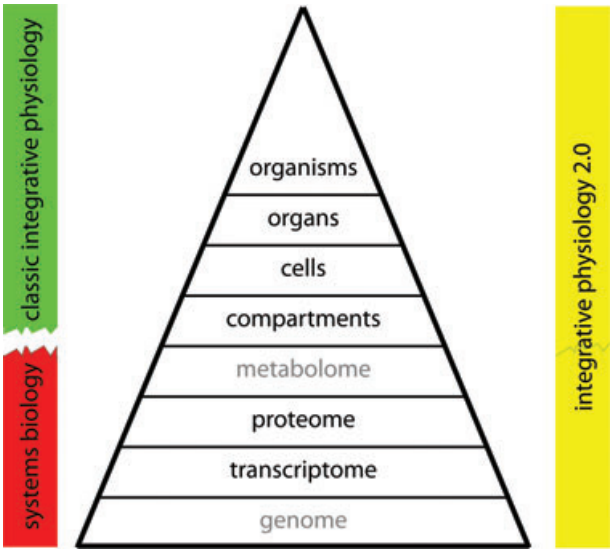

Figure 1. From systems biology and classical integrative physiology towards Integrative Physiology 2.0
A process such as cardiac remodelling should be studied at different levels and the findings integrated. The bars on the left illustrate the dichotomy between classic integrative physiology and systems biology. The bar on the right illustrates the 'Integrative physiology 2.0' approach, which integrates the large scale unbiased '-omics' studies of systems biology with integrative physiology. Levels shown with a grey font have not been studied by our group, to date.

In this article we will present studies on the adjustments of the myocardium to acute and chronic increases in loading conditions, in order to highlight the established strengths of classical integrative physiology and the promise of integrating systems biology and physiology. We begin to review our studies using classical *in vivo* physiology approaches to study regulation of cardiac function and coronary blood flow in response to acute exercise. We will then discuss how we have implemented biochemistry, molecular biology, and more recently bioinformatics to study biological processes in a more holistic rather than reductionistic fashion to understand complex processes such as cardiac remodelling and hypertrophy.

Plasticity of the cardiovascular system: acute responses to exercise

One of the most dramatic challenges for the cardiovascular system is represented by sudden heavy physical exercise, requiring both central and regional haemodynamic adjustments in order to meet increases in metabolic needs of skeletal and cardiac muscle. A fivefold increase in cardiac output together with a redistribution of flow away from visceral organs and tissues is needed to accommodate sufficient increases in skeletal muscle and myocardial blood flow. The increases in muscle blood flow are facilitated by a small increase in aortic blood pressure but are opposed by the compressive forces generated by the contracting muscle, acting on the intramuscular vasculature. Consequently, the increases in flow are principally due to vasodilatation of the resistance vessels within the skeletal and cardiac muscle.

A large number of vascular control mechanisms have been identified that can contribute to metabolic regulation of resistance vessel tone in the heart and skeletal muscle (Fig. 2), including blood-derived, endothelial, metabolic and sympathetic influences. However, unravelling of the exact mechanism that mediates the exercise-induced vasodilatation has proven to be difficult (Laughlin *et al.* 1996; Rowell, 2004; Tune *et al.* 2004; Duncker & Merkus, 2007; Duncker & Bache, 2008). Since maintenance of tissue perfusion is essential for adequate cardiac and skeletal muscle function and organismal survival, it is not surprising that regulation of tissue blood flow is characterized by a high number of redundant control mechanisms (Rowell, 2004; Duncker & Bache, 2008). A consequence of this non-linear redundancy design is that pharmacological blockade of a single vasodilator mechanism may have little or no effect (and may thus not reveal the actual contribution of that mechanism), as other vasodilator pathways will increase their activity and act to compensate. Only when multiple pathways are blocked will an effect become apparent, which is then greater than the sum of the effects of blocking the individual pathways. Indeed, studies in cardiac and skeletal muscle have demonstrated that simultaneous blockade of various vasodilator substances was required to attenuate the increase in skeletal muscle flow (Murrant & Sarelius, 2002; Boushel, 2003) or coronary blood flow (Duncker & Bache, 2008) during exercise. These observations demonstrate the importance of an integrative approach looking at the whole system and the interaction between the individual components.

Plasticity of the cardiovascular system: cardiac remodelling after myocardial infarction

The cardiovascular system is not only able to respond quickly to acute challenges, but also has the plasticity to respond to chronic changes in haemodynamic loading conditions, for example as occurs following an acute myocardial infarction (MI). Loss of a significant portion of myocardial tissue results in an immediate decrease in cardiac pump function, leading to neurohumoral activation that is aimed at restoring pump function.

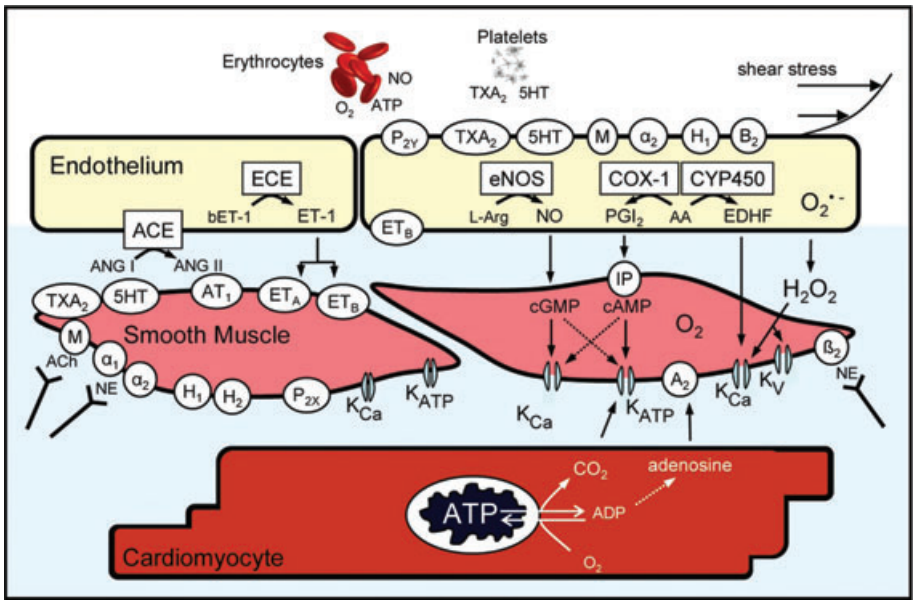

Figure 2. Schematic drawing of the various influences that determine coronary vasomotor tone and diameter

Influences include autonomic nervous system activity, metabolic factors from cardiomyocytes and endothelial factors. The latter are modified by physical forces (shear stress), as well as erythrocyte and platelet-derived products acting on the endothelium. TxA_2, thromboxane A_2 (receptor); 5HT, serotonin or 5-hydroxytryptamine (receptor); P_{2X} and P_{2Y}, purinergic receptor subtypes 2X and 2Y that mediate ATP-induced vasoconstriction and vasodilatation, respectively; ACh, acetylcholine; M, muscarinic receptor; H_1 and H_2, histamine receptors type 1 and 2; B_2, bradykinin receptor subtype 2; ANG I and ANG II, angiotensin I and II; AT_1, angiotensin II receptor subtype 1; ET, endothelin; ET_A and ET_B, endothelin receptor subtypes A and B; A_2, adenosine receptor subtype 2; β_2, β_2-adrenergic receptor; α_1 and α_2, α-adrenergic receptors; NO, nitric oxide; eNOS, endothelial NO synthase; PGI_2, prostacyclin; IP, prostacyclin receptor; COX-1, cyclooxygenase-1; EDHF, endothelium-derived hyperpolarizing factor; CYP_{450}, cytochrome P_{450} 2C9; K_{Ca}, calcium-sensitive K^+ channel; K_{ATP}, ATP-sensitive K^+ channel; K_V, voltage-sensitive K^+ channel; AA, arachidonic acid; L-Arg, L-arginine; O_2^-, superoxide. Receptors and enzymes are indicated by an oval and rectangle, respectively. From Duncker & Bache (2008), modified with permission from the American Physiological Society.

The neurohumoral activation results in a wide array of responses varying from the immediate (seconds–minutes) positive chronotropic, inotropic and lusitropic cardiac effects and sub-acute (hours–days) volume retention, to the chronic (days–months) cardiac remodelling, characterized by hypertrophy of the cardiac muscle (Katz, 2003). All these responses aim to maintain pump function of the injured heart. However, despite the apparent appropriateness of the hypertrophic remodelling response to maintain cardiac pump function early after MI (van Kats *et al.* 2000), hypertrophic remodelling constitutes an independent risk factor for the long-term development of congestive heart failure (Levy *et al.* 1990; Vakili *et al.* 2001). The mechanism underlying progressive deterioration of left ventricular (LV) function towards overt heart failure remains incompletely understood, but may involve (i) continuous loss of cardiomyocytes through apoptosis (Narula *et al.* 2006), (ii) a primary reduction in contractile function of the surviving myocardium (van der Velden *et al.* 2004), (iii) alterations in extracellular matrix leading to progressive LV dilatation (Spinale, 2007), and/or (iv) myocardial blood flow abnormalities, resulting in impaired myocardial O_2 delivery to the non-infarcted region (van Veldhuisen *et al.* 1998). Blood flow to the remodelled myocardium can become impeded as the coronary vasculature does not grow commensurate with the increase in LV mass and because extravascular compression of the coronary vasculature increases with increased LV filling pressures (Haitsma *et al.* 2001). In addition, an increase in coronary resistance vessel tone, secondary to neurohumoral activation and endothelial dysfunction, could also contribute to the impaired myocardial oxygen supply.

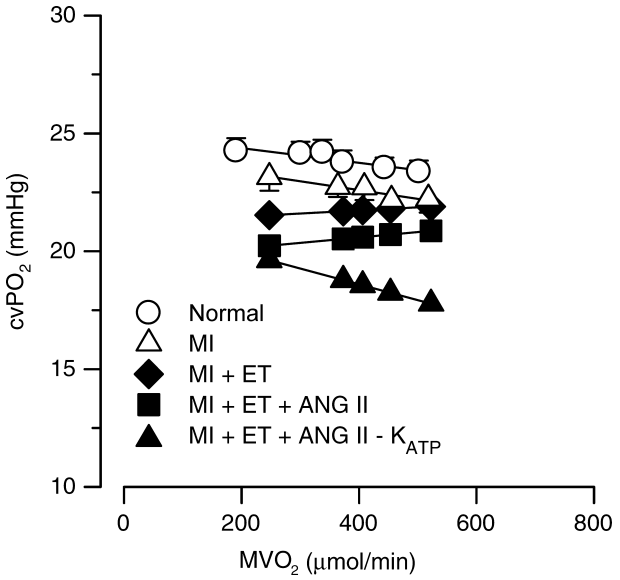

Figure 3. Myocardial oxygen balance in normal and MI swine
Shown are the relations between myocardial oxygen consumption ($M\dot{V}_{O_2}$) and coronary venous oxygen tension (cvP_{O_2}) in 30 normal swine (open circles) and 20 MI swine (open triangles) under control conditions. Data were obtained at rest and during increases in $M\dot{V}_{O_2}$ produced by graded treadmill exercise (1–5 km h^{-1} in normal swine and 1–4 km h^{-1} in MI swine). In addition, we have depicted the computed relations in MI swine if the ET (filled diamonds) and ANG II (filled squares) vasoconstrictor influences (which were both attenuated in MI swine) and the K_{ATP} (filled triangles) vasodilator influences (which were enhanced in MI swine) would have been identical to those in normal swine. The graph clearly illustrates that the adaptations in coronary vasomotor control act to blunt perturbations in oxygen balance in remodelled myocardium of swine with a recent MI. Modified from Duncker *et al*. (2008) with permission from Springer Science+Business Media.

Consequently, we explored in a series of studies the alterations in regulation of coronary resistance vessel tone in post-MI remodelled myocardium. For this purpose we employed a porcine model of MI produced by permanent ligation of the left circumflex coronary artery, which results in transmural infarction of 20–25% of the LV free wall, and studied swine at 2–3 weeks after induction of MI. Swine were not only studied at rest but also during graded treadmill exercise to further stress the remodelled hearts and recruit the cardiac and coronary functional reserve capacity, to facilitate elucidation of compensatory mechanisms that become activated to maintain cardiovascular homeostasis. These studies indicate that myocardial oxygen balance is mildly perturbed in remodelled myocardium. Thus at a similar level of cardiac work and hence oxygen consumption, coronary blood flow and hence myocardial oxygen supply are lower in MI compared to normal swine, forcing the myocardium to increase its oxygen extraction leading to a lower coronary venous oxygen content (Fig. 3). That the relatively small degree of perturbation in the oxygen balance was associated with myocardial metabolic distress was also reflected in the increased vasodilator influence through opening of K_{ATP} channels, particularly during exercise (Merkus *et al.* 2005b). Unexpectedly, we observed that despite increased circulating levels of noradrenalin, angiotensin II and endothelin-1, the coronary influences of α-adrenergic tone were not increased (Duncker *et al.* 2005), while the coronary vasoconstrictor influences of endogenous endothelin (Merkus *et al.* 2005*a*) and angiotensin II (Merkus *et al.* 2006) were virtually abolished. Thus, early after myocardial infarction, small perturbations in myocardial oxygen balance were observed in remodelled myocardium. However, adaptations in coronary resistance vessel control, consisting of increased vasodilator influences in conjunction with blunted vasoconstrictor influences, acted to minimize the impairments of myocardial oxygen balance (Fig. 3). These studies not only highlight the plasticity of the post-MI remodelled heart and coronary circulation, to minimize perturbations in myocardial oxygenation in the face of increased compressive forces and reduced capillary densities, but also illustrate the necessity

to study these phenomena in an integrative manner in an intact animal model.

Neurohumoral activation following MI initially contributes to circulatory homeostasis, but will eventually contribute to the progressive deterioration in LV function. This concept is supported by studies showing detrimental effects of amplification of neurohumoral activity by phosphodiesterase-3 (PDE3) inhibitors in patients with heart failure (Packer *et al.* 1991), while on the other hand β-adrenergic receptor blockade (CIBIS Investigators and Committees, 1994; CIBIS-II Investigators and Committees, 1999; MERIT-HF Study Group, 1999) and inhibitors of the RAAS system (Pfeffer *et al.* 1992) have clearly shown long-term benefits in large cohorts of patients with heart failure. Starting from these observations in patients with heart failure, we took an integrative approach to study the cellular and molecular mechanisms underlying LV dysfunction observed in our swine model ~3 weeks after acute MI. In a first series of studies, we demonstrated the presence of LV remodelling (van Kats *et al.* 2000) and dysfunction (Duncker *et al.* 2001; Haitsma *et al.* 2001), necessitating an increased oxygen extraction by the peripheral tissues (Fig. 4*A*) and causing an increase in neurohumoral activation (Fig. 4*B*) (Haitsma *et al.* 2001). Despite the increased neurohumoral activation, β-adrenergic inotropic (Fig. 4*C*) and lusitropic (Fig. 4*D*) influences on the left ventricle were markedly blunted, particularly during treadmill exercise (van der Velden *et al.* 2004; Duncker *et al.* 2005). A loss of β-adrenergic signalling was also suggested by an attenuated response to PDE3 inhibition (Duncker *et al.* 2001). To further investigate the cellular mechanisms underlying the global LV dysfunction, we performed studies in isolated permeabilized individual cardiomyocytes (van der Velden *et al.* 2004). In myocytes from the remote LV zone in MI hearts, we observed abnormalities in myofilament force development, which correlated well with the degree of LV remodelling, and an increase in myofilament Ca^{2+} sensitivity (Fig. 5*A*) (van der Velden *et al.* 2004). These alterations in myofilament function are likely to contribute to the systolic (Fig. 4*C*) and diastolic (Fig. 4*D*) LV dysfunction observed in swine during β-adrenergic receptor activation produced by treadmill exercise. The abnormalities in myofilament function could be prevented, at least in part, by treatment with chronic β_1-adrenergic receptor blockade during the post-MI period (Duncker *et al.* 2009). Analysis of myofilament proteins with one- and two-dimensional gel-electrophoresis failed to demonstrate significant alterations in phosphorylation status under basal conditions, including to our surprise the β-adrenergic target proteins cardiac myosin binding protein C (Fig. 5*C*) and troponin I (Fig. 5*D*) (Duncker *et al.* 2009). When the heart was stimulated with the β-adrenergic receptor agonist dobutamine, the increase in troponin I phosphorylation was blunted in remodelled myocardium (Fig. 5*D*) (Boontje *et al.* 2010). The increased Ca^{2+} sensitivity of force development of post-MI myocytes could be restored to normal (sham) values by incubation with the catalytic subunit of protein kinase A (PKA), the downstream kinase of the β_1-adrenergic receptor (Fig. 5*B*). Taken together, these observations suggest that PKA specific phosphorylation sites may be selectively altered in post-MI hearts, which are the subject of ongoing studies within our laboratory.

To complement the top-down approach (from organism towards proteome) outlined above and to further investigate the mechanisms underlying the LV dysfunction following MI, we recently set out to investigate transcriptional control of LV remodelling and dysfunction. For this purpose, we performed microarray analysis to find genes that are differentially expressed in post-MI *versus* control hearts (Kuster *et al.* 2010). Relations between the differentially expressed genes were assessed by Ingenuity Pathway Analysis. This program

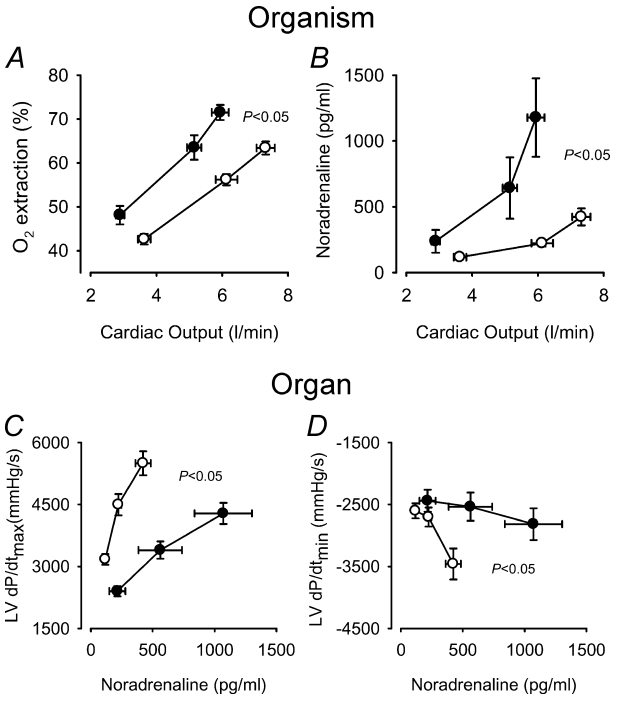

Figure 4. Functional changes at the whole-body and cardiac level
Whole-body oxygen extraction (*A*) and circulating noradrenaline levels (*B*) in resting and exercising swine with cardiac dysfunction 3 weeks after MI (filled circles) or sham surgery (open circles), and maximum rates of rise (*C*) and fall (*D*) of left ventricular pressure were plotted as a function of circulating noradrenaline levels. In each group, data are shown during resting conditions and during treadmill running at 2 and 4 km h^{-1}. The data show relatively little functional deficit in resting conditions, but functional deficits at higher endogenous sympathetic activation increasing with exercise intensity. Based on Haitsma *et al.* (2001) with permission from the European Society of Cardiology and van der Velden *et al.* (2004).

builds networks of interacting molecules by connecting as many differentially expressed genes as possible, and allowing for hub molecules of which the expression remains unchanged. Taken a non-supervised approach (Fig. 6*A*), an important network was identified that contained several genes encoding proteins involved in β-adrenergic signalling, including the regulatory subunit of PKA (PRKAR2B), A-kinase anchoring protein 5 (AKAP5), calmodulin and calmodulin kinase (CaMK), of which the expression was altered. In addition, subsequent analysis of the β-adrenergic signalling network revealed increased expression of PDE4 (Fig. 6*B*). If confirmed at the protein level, the increased expression could contribute to the observed blunted PKA influence on myofilament Ca^{2+} sensitivity via (i) reduced cAMP production through increased CaMK-mediated inhibition of adenylyl-cyclase and increased cAMP breakdown by PDE4, and (ii) inactivation of the catalytic subunit of PKA by increased binding to the regulatory subunit of PKA.

Integrative Physiology 2.0

Systems biology approaches have not yet been applied to the study of cardiac remodelling, largely because of its tremendous complexity. Starting from observations in

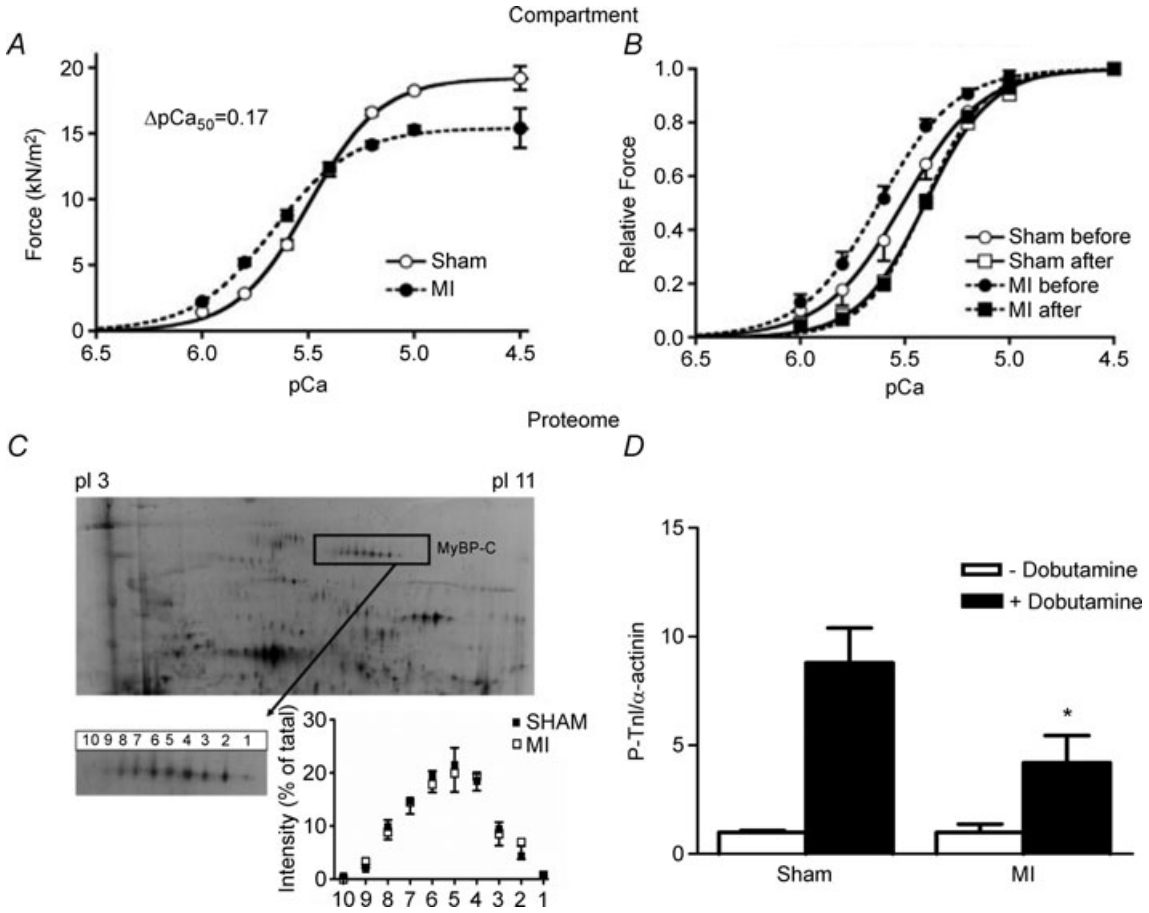

Figure 5. Myofilament function and protein phosphorylation
A, determination of force development by skinned cardiomyocytes isolated from sham and post-MI pig hearts at different exogenous Ca^{2+} concentrations showed reduced maximal force and increased Ca^{2+} sensitivity in post-MI remodelled myocardium. *B*, Ca^{2+} sensitivity in the MI hearts was normalized to control (sham) values by pre-incubation of skinned cardiomyocytes with exogenous protein kinase A (PKA). Force development was measured before and after incubation with PKA. Force at maximal [Ca^{2+}] was set to 1. The observation that PKA abolished the difference in Ca^{2+} sensitivity between sham and post-MI cardiomyocytes suggests that the increase in myofilament Ca^{2+} sensitivity is caused by lower levels of PKA-mediated phosphorylation of sarcomeric proteins. *C*, two-dimensional gel electrophoresis showed no difference in the phosphorylation pattern of the PKA target protein cardiac myosin binding protein (cMyBP-C) between sham and MI hearts. *D*, troponin I (TnI) phosphorylation did not differ under baseline conditions between sham and MI heart. However, following intravenous infusion of dobutamine the increase in TnI phosphorylation was attenuated in post-MI myocardium. Panels *A* and *B* were adapted from van der Velden *et al.* (2004), *C* was adapted from Duncker *et al.* (2009) and *D* shows data from Boontje *et al.* (2010).

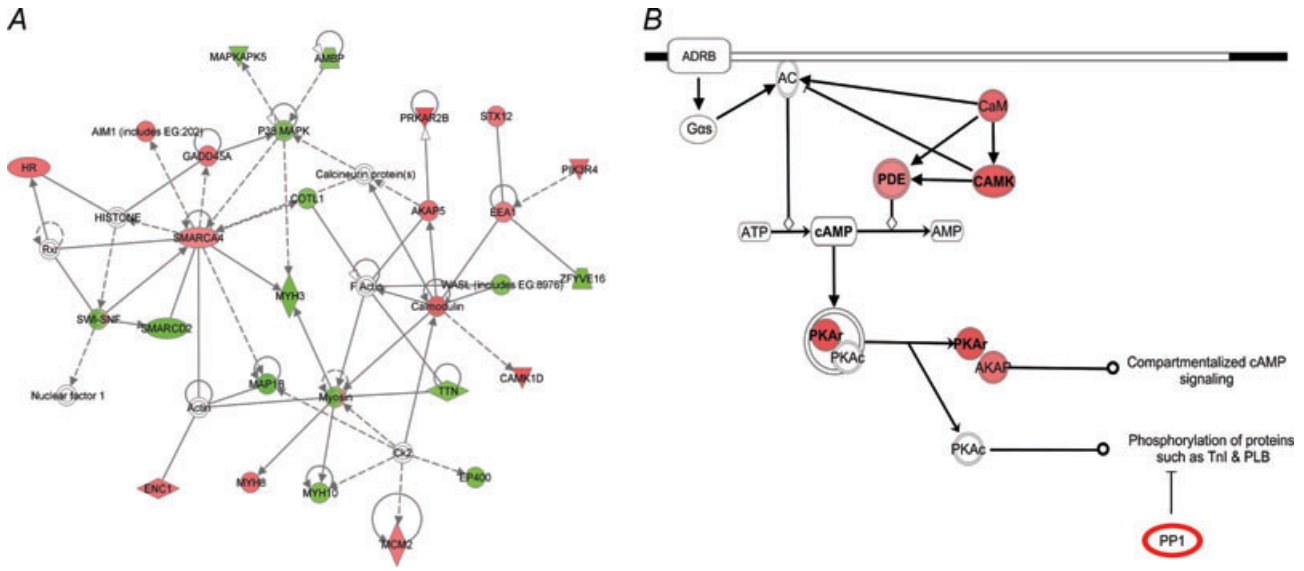

Figure 6. Network identification by Ingenuity Pathway Analysis
A, one of the major networks identified by unsupervised analysis of genes differentially expressed in post-MI *vs.* sham myocardium. Genes in red and green are up- and downregulated after MI, respectively. Genes in white, such as calcineurin, are not changed in expression but represent hubs between a large number of differentially expressed genes. The data show that a number of genes of the β-adrenergic pathway are changed in expression. *B*, simplified β-adrenergic signalling pathway identified by supervised data analysis, with upregulated genes in red and downregulated genes in green. Colour intensities correspond to the degree of change, with a deeper colour indicating a greater change. PP1 has been depicted with a red outline to indicate that we previously found an increase in PP1 protein level. Data are from Kuster *et al.* (unpublished).

patients showing detrimental effects of PDE3 inhibitors and beneficial effects of β-blockers, we have taken an integrative approach to studying the mechanisms underlying LV dysfunction after MI (Fig. 7). We began by narrowing our experimental focus to the well-defined clinical phenotype of post-MI LV remodelling and took a top-down approach, starting in the awake pig and ending with specific and generalized molecular investigations

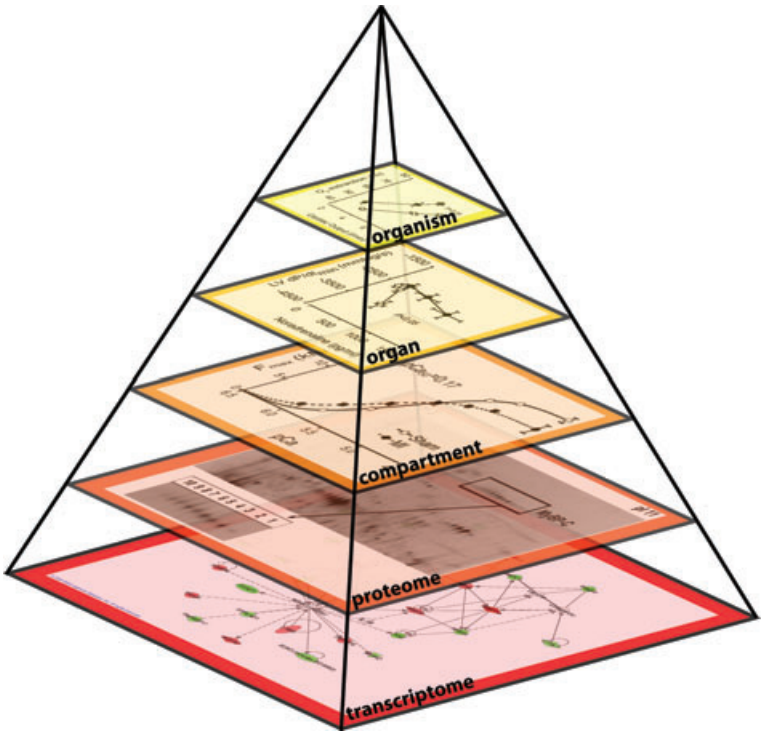

Figure 7. Illustration of our 'Integrative Physiology 2.0' approach
Complex physiological processes such as cardiac remodelling must be studied in detail at different levels ranging from the transcriptome of cells all the way up to the intact organism, and possibly even further to population-based functional responses to pharmacons (not shown). At each level, data should be integrated with 'higher' and 'lower' levels, to build a multidimensional picture of the ongoing processes.

centred on transcriptomic and proteomic correlations (Fig. 7) based on current knowledge (Adams, 2010). Using a porcine model of post-MI remodelling, we first demonstrated the presence of LV remodelling and pump dysfunction in swine, necessitating increased oxygen extraction by the peripheral tissues and causing an increase in neurohumoral activation (*organism*). Despite the increased neurohumoral activation, β-adrenergic receptor mediated increases of LV function (*organ*) were blunted (Duncker *et al.* 2005), which coincided with attenuated LV inotropic responses to PDE3 inhibition (Duncker *et al.* 2001). Further studies at the cardiomyocyte level revealed abnormalities of myofilament force development that correlated well with the degree of LV remodelling (*cellular compartment*) (van der Velden *et al.* 2004). The alterations in myofilament Ca^{2+} sensitivity appeared to be mediated by loss of PKA catalytic activity (*proteome*), and could be prevented by simultaneous treatment with β_1-adrenergic receptor blockade, coinciding with an improvement in LV pump function (Duncker *et al.* 2009). Non-supervised as well as supervised network analysis of microarray data (*transcriptome*) revealed significant alterations in expression of genes encoding proteins involved in β-adrenergic receptor signalling (Fig. 7). These preliminary findings will be followed up by further studies into translational and post-translation modifications.

Since the completion of the Human Genome Project and the advent of the large scaled unbiased '-omics' techniques, the field of systems biology has emerged. Systems biology aims to move away from the traditional reductionist molecular approach, which focused on understanding the role of single genes or proteins, towards a more holistic approach by studying networks and interactions between individual components of networks. From a conceptual standpoint, systems biology elicits a 'back to the future' experience for any integrative physiologist, and we feel that systems biology can benefit from the knowledge and existing models of interaction between systems available in physiology. Conversely, many of the new techniques and modalities employed by systems biologists yield tremendous potential for integrative physiologists to expand their tool arsenal to (quantitatively) study complex biological processes, such as cardiac remodelling and heart failure, in a truly holistic fashion. Such an approach may generate new hypotheses, concepts and eventually novel treatments for the process of cardiac remodelling and heart failure, which should subsequently be tested in a physiological setting. We therefore advocate that systems biology should not become/stay a separate discipline with '-omics' as its playing field, but should be integrated into physiology to create 'Integrative Physiology 2.0', allowing interconnection and integration of processes at the various levels of complexity and organization within the pyramid of life.

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