Review

Signal transduction revisited: recent developments in angiotensin II signaling in the cardiovascular system

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1. Introduction

Two years have passed since the appearance of the focused issue on signal transduction (Cardiovascular Research, volume 30, issue 4, October 1995). In that issue a selection of topics related to cardiovascular signal transduction were highlighted. Since then a wealth of new information has deepened our insight into the factors that govern the activity of the different signal transduction pathways. Moreover, the importance of cross-talk between signal transduction pathways is being recognized and has, at the same time, made us aware of the complexity of the signal transduction network. Finally, new players in the field of cardiovascular signal transduction have emerged, the most striking example being the Stress-Activated Protein Kinases.

The purpose of this review is to summarize these new developments in this area of research. Considering the rate with which the various signal transduction pathways and their interrelationships are being deciphered the task of writing an update that pays tribute to all these new insights and developments is virtually impossible. Accordingly, as a major theme of this review the recent developments on angiotensin II-mediated signal transduction was chosen. This choice is not entirely arbitrary. In the October 1995 issue on cardiovascular signaling the role of angiotensin II (Ang II) was a main topic of various reviews already [1–3]. More importantly Ang II, either systemic or locally produced, is considered to play a pivotal role in cardiovascular adaptation. Signaling via this peptide growth factor involves various receptor subtypes and results in the activation of a variety of signal transduction cascades, which have been unravelled to a large extent over the past couple of years.

2. Mechanical forces and signal transduction

The major type of mechanical force to which the cardiac muscle is subjected is cyclic stretch. In contrast, the vascular endothelial cells face both cyclic stretch (pressure) and shear forces (blood flow). It is generally accepted that mechano-sensing is of paramount importance in the activation of signal transduction pathways in endothelial cells, vascular smooth muscle cells, and cardiac muscle cells, that enable the cardiovascular system to adjust to changing demands [4,5]. Nevertheless the precise mechanism of cellular mechano-sensing remains to be elucidated. To date three major hypotheses have been put forward to pinpoint the molecular basis of mechano-sensing. According to the first hypothesis mechanical stimuli are sensed by stretch-activated ion channels [6,7]. Patch clamp analysis has shown that these channels behave like non-selective cation channels that can be blocked by gadolinium. Second it has been proposed that the molecular components of the extracellular matrix (ECM) and cytoskeleton are responsible for mechano-sensing [8]. In this context the role of the family of proteins referred to as integrins and so-called focal adhesion kinases (FAK) have received widespread attention [9,10]. The integrins have been thought to act as transmembrane mechanoreceptors. FAK possesses tyrosine kinase activity and becomes activated when a cell makes contact with the extracellular matrix [11]. Whatever the nature of the mechano-receptors, there is ample evidence to support the notion that

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mechano-transduction provides the initial trigger for short-term (cardiac contractility, vascular tone) as well as long-term adaptive responses (cardiac and vascular remodeling) in the cardiovascular system.

The third hypothesis is based on the experimental observation that applying a mechanical stimulus to most cell types leads to the synthesis of a whole range of autocrine and paracrine factors that, in turn, are able to modulate the activity of various signal transduction pathways. Hence, according to this hypothesis the question as to how mechanical signals are conveyed to the cells interior, can be narrowed down to the question as to how the synthesis of these growth factors is induced in response to mechanical stimuli. In this sense the latter hypothesis does not provide a true mechanistic explanation for mechano-sensing.

Imposing shear stress to endothelial cells has been shown to lead to the formation of, amongst others, endothelin-1 (ET-1), basic fibroblast growth factor (bFGF), and Nitric Oxide (NO) [12,13]. Stretch of cardiac myocytes has been shown to induce the synthesis and release of Angiotensin II (Ang II) [7]. In addition, increased synthesis of transforming growth factor-β (TGFβ) and endothelin-1 (ET-1), either directly as a result of the mechanical stimulus or secondary to the effects of stretch-induced Ang II production, have been reported [14,15]. Each of these factors has been shown to induce changes in gene expression in primary cultures of neonatal cardiac myocytes that are reminiscent of the hypertrophic response of the heart in situ [16,17].

3. Systemic and local angiotensin II

Ang II is the biologically most active component of the renin angiotensin system. The physiological effects of circulating Ang II, such as its involvement in blood pressure control, aldosterone release and water balance, are relatively well known. Only recently the biological significance of locally produced Ang II is being acknowledged. It has become apparent that the cardiac tissue itself expresses all components of the renin angiotensin system, i.e. renin, angiotensinogen, and angiotensin converting enzyme and that the expression of these components alters under pathophysiological conditions, like cardiac hypertrophy [18]. Induction of cardiac hypertrophy in rats in situ by supra-renal constriction of the aorta has been shown to increase circulating levels of Ang II, in addition to the upregulation of the expression of components of the renin angiotensin system in the heart, suggesting activation of the intracardiac renin angiotensin system [19]. In addition, cyclic stretch of cardiac myocytes has been shown to upregulate the activity of the angiotensinogen promoter [20] and elicits release of Ang II into the surrounding medium [7]. These findings support the notion that the intracardiac renin angiotensin system is of functional importance. The biological significance of Ang II is further illustrated by the fact that addition of a specific type I Angiotensin receptor antagonist to the culture medium prevents the effects of mechanical stimuli on cardiomyocyte phenotype [21]. Accordingly, when evaluating the effects of Ang II in situ both changes in circulating levels and local production have to be taken into account. Divergent effects of Ang II at the systemic and tissue level have been reported by the group of Delafontaine [22,23], who showed that venous infusion of Ang II in rats reduces Insulin like Growth Factor 1 (IGF-1) levels in the circulation, while at the same time increasing IGF-1 mRNA levels in the heart.

4. Angiotensin II receptors

Ligand binding studies have revealed the existence of at least two Angiotensin II receptor subtypes, referred to as the angiotensin receptor type I (AT₁) and type II (AT₂), respectively. Recently the existence of another subtype in human cardiac fibroblasts has been postulated on the basis of its pharmacological profile in binding studies with AT₁ and AT₂ specific ligands [24]. The AT₁ and AT₂ receptors have been cloned and appeared to be members of the G-protein-coupled seven-transmembrane-domain receptor superfamily, which also includes the α- and β-adrenergic receptors and the endothelin receptors. Both AT₁ and AT₂ receptors are present in the heart, although with respect to their relative densities species differences have to be appreciated. Within the rat heart cardiomyocytes and fibroblasts have been demonstrated to express the AT₁ receptor subtype mainly, whereas endothelial cells possess AT₁ as well as AT₂ receptors [25]. To date virtually all of the physiological effects of Ang II are ascribed to the AT₁ receptor. As described in detail below the signaling cascades involving the AT₁ receptor have been largely elucidated (see Berk and Corson [26] for recent review). However, relatively little is known about the coupling of the AT₂ receptor to intracellular signaling pathways. In this respect it is worth mentioning that the AT₂ receptor is the predominant receptor subtype found in the human heart [27,28].

It has been demonstrated that various (pathological) stimuli lead to a shift in AT₁ and AT₂ receptor density. However, as far as the direction of this shift is concerned, the results reported are not very consistent. In cultured neonatal myocytes and fibroblasts incubation with Ang II resulted in a downregulation of AT₁ mRNA levels [29]. In another study [21] it was demonstrated that stretching of neonatal myocytes, which supposedly also leads to Ang II secretion by these cells [14], is accompanied by a simultaneous upregulation of AT₁ and AT₂ receptor mRNA.

Similarly, at the tissue level conflicting results have been reported. Right ventricular hypertrophy and failure in the canine heart was associated with local increases of the mRNA level of ACE and the AT₁-receptor, whereas the AT₁ receptor level was decreased [30]. Using rat models of
cardiac hypertrophy and failure various groups were unable to detect changes in AT1 or AT2 receptor mRNA levels [31,32], whereas others reported downregulation [33,34] or upregulation [25] of the AT1 receptor. Also in the human failing heart the AT1 receptor mRNA level was found to decline, whereas that of the AT2 receptor was unaltered [35]. The discrepancies observed are probably related to species differences and to differences in the experimental models used (pressure- versus volume-overload, compensated versus decompensated hypertrophy, etc.). In addition, it is feasible that time-dependent changes in receptor-subtype expression are also involved. Whatever the exact cause, these findings indicate that the modulation of angiotensin II receptor subtype density, and consequently the activation of partly different downstream signal transduction pathways, provides another level of regulation with respect to Ang II signaling. Further complexity is added to the system by AT receptor desensitization, which has been shown to occur rapidly following ligand binding [36]. Circumstantial evidence indicates that protein kinase C (PKC)-mediated phosphorylation events may be involved in the desensitization of receptors. However, a role of PKC in AT receptor desensitization could not be demonstrated [36].

5. The AT2 receptor

Given the predominance of AT2 receptors in the human heart [27] and the observed changes in relative abundance of the AT1 and AT2 receptor subtypes under various pathological conditions (described above) a more detailed knowledge of the AT2 receptor and its downstream signaling pathways is of primary importance. However, current knowledge on AT2 receptor-mediated signaling is scarce [37]. Despite its structural identification as a G-protein-coupled seven-transmembrane receptor, analysis of putative downstream signaling pathways in human embryonic kidney cells overexpressing the AT2 receptor did not provide evidence for its coupling to typical G-protein mediated signaling mechanisms [38]. However, more recent findings based on immunohistochemical techniques indicate that the AT2 receptor specifically interacts with Goi [39]. Rabkin [40] provided evidence that the AT2-receptor might be linked to protein kinase C (PKC), as the translocation of PKC from the soluble to particulate fraction was inhibited by PD123319, a specific AT2-receptor antagonist. Ang II-induced elevation of the intracellular pH in neonatal cardiac myocytes could be blocked with the AT2-receptor antagonist PD123319, but not with the AT1-receptor antagonist Losartan [41]. Furthermore, alkalization was also observed when the cells were superfused with arachidonic acid, thereby suggesting that activation of phospholipase A2, via the AT2-receptor might be involved. Recently, Booz and Baker [42] observed that AT1 receptor antagonists prevented the Ang II-induced elevation of protein synthesis, while AT2 antagonists actually potentiated the Ang II-induced $[^{3}H]$leucine incorporation. These and other studies strongly suggest that the AT1 and AT2 receptors may serve opposite functions [43]. In fact it has been postulated that the therapeutic effect of AT1 receptor antagonists in the setting of cardiac failure may be due in part to the specific activation of AT2 receptors secondary to the increase in renin and angiotensins [44].

6. Signaling through the AT1 receptor

Recent studies have revealed a wide variety of downstream signaling cascades that are activated by AT1-receptor occupancy, including the ‘classical’ GTP-binding protein (G-protein)–phospholipase C (PLC) cascade, and various pathways involving tyrosine kinases. The principal features of each of these pathways are outlined below. Again it is important to stress that presenting these pathways as individual cascades is necessary for the sake of clarity, but does not pay tribute to the complexity of the cellular response, in which various factors involved in ‘one’ cascade are part of, and/or influence, other cascades.

6.1. Involvement of different phospholipase C-isoforms

To date as many as 10 phosphoinositide-specific phospholipase C (PLC) isozymes have been identified. These isozymes can be subdivided into three subclasses, i.e., PLC-β, PLC-γ and PLC-δ on the basis of sequence homologies and certain motifs in their catalytic domain [45]. The mode of activation of the δ-isozymes is largely unknown. The activity of PLC-β isozymes is modulated by G-proteins [46], whereas PLC-γ isozymes contain so-called SH2 (Src-homology) domains that allow them to interact with tyrosine phosphorylated receptors [47]. The functional importance of the different isoforms is illustrated by the fact that knock-out of the gene encoding PLC-γ1 resulted in embryonic lethality, thereby demonstrating that at this developmental stage other PLC-isoforms obviously can not functionally compensate for this specific isozyme [48].

As the AT1 receptors are G-protein-coupled receptors it was first believed that PLC-β was the isozyme most likely involved in the downstream signaling pathways (Fig. 1). However, recent findings strongly support the notion that, in addition to PLC-β, PLC-γ is an important downstream effector of the AT1 receptor. As the AT1 receptor lacks intrinsic tyrosine kinase activity, the issue of how this type of receptors is linked to tyrosine kinase pathways has been a key question, which by now has largely been solved (see below).

6.2. G-protein / PLC-β mediated signaling

The role of G-proteins in cardiac signal transduction was extensively reviewed by Brodde et al. [49]. The
G-proteins act as coupling proteins that become activated upon receptor stimulation. In its GDP-bound form, the protein forms an inactive heterotrimer composed of an α-, β-, and γ-subunit associated with the receptor. Upon ligand binding, conformational changes in the receptor are transmitted to the α-subunit resulting in the release of GDP and its replacement by GTP. Subsequently, the α- and the βγ-subunits become dissociated and are released from the receptor. Both the α-subunit and βγ-subunit are able to activate target systems [50]. As reviewed by Brodde and coworkers [49] the G-proteins are classified on the basis of their Gα-subunit. Three major subfamilies of G-proteins can be distinguished, Gox, Goxi, and Goxq, which couple to different sets of receptors. The AT₁, α₁-adrenergic and endothelin-1 receptor make use of Gox1. In the case of the α₁-adrenergic receptor, this has been convincingly shown by demonstrating that microinjection of neutralizing antibodies against Goxq suppressed the stimulatory effect of α₁-adrenergic stimulation on atrial natriuretic factor (ANF) expression in neonatal cardiomyocytes [51].

Thus the binding of Ang II to the AT₁ receptor elicits the release of GoxqGTP which activates PLC-β which, in turn, catalyzes the hydrolysis of phosphatidylinositol-4,5-biphosphate into inositol-1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG) (Fig. 1). IP₃ stimulates the release of Ca²⁺ from intracellular stores. Although the importance of IP₃ in cellular Ca²⁺ signaling has been established for a large number of cell types, its significance in signal transduction in the cardiomyocyte remains controversial [52]. However, the importance of the lipid second messenger DAG in the activation of PKC is without question.

![Fig. 1. Schematic presentation of the two pathways that couple the AT₁ receptor to PKC. Following ligand binding the G-protein coupled AT₁ receptor activates PLC-β via the Gαq subunit. PLC-γ becomes tyrosine phosphorylated and activated by the kinase Src. Activation of both PLC isozymes leads to IP₃ and diacylglycerol formation and the subsequent activation of PKC. One of the downstream targets of PKC is the Raf–MAPK pathway (see also Fig. 2). Abbreviations are explained in the text.](image)

### 6.3. The AT₁ receptor / PLC-γ connection

The virtual absence of PLC-β in cultured vascular smooth muscle cells (VSMC) raised doubts about its role in IP₃ formation subsequent to Ang II application in this cell type [53]. Nevertheless, Ang II application led to a rapid production of IP₃. Furthermore, inhibitors of tyrosine kinases blocked the Ang II induced IP₃ formation in VSMC. These and other findings point to a functional link between the AT₁ receptor and PLC-γ. Indeed, stimulation of VSMC with Ang II resulted in the tyrosine phosphorylation of PLC-γ1. Just recently the molecular link between G-protein-coupled receptors and tyrosine phosphorylation of PLC has been elucidated. Members of the Src-family of tyrosine kinases were considered likely candidates as in vitro studies had already shown that PLC-γ is a good substrate for this family of kinases [54]. Subsequent studies have provided firm evidence that Src is part of the pathway that couples Ang II with PLC-γ. It has been shown that Ang II administration leads to a rapid activation of Src in VSMC [55] and that anti-Src antibodies, introduced into the intracellular milieu of VSMC by electroporation, substantially reduced PLC-γ phosphorylation and IP₃ production following Ang II stimulation [56]. In a recent paper Schelling and coworkers [57] challenged the notion that PLC-γ is the principal PLC isozyme involved, as they were able to demonstrate the presence and functional significance of PLC-β1 in vascular smooth muscle cells of rat and human origin.

### 6.4. Protein kinase C

PKC’s are serine/threonine kinases that can be classified into those isoforms that are phosphatidylinerine, Ca²⁺, DAG or phorbolester-dependent (the ‘classical’ cPKC α, β₁, β₂, γ), those that are Ca²⁺-independent (the ‘novel’ nPKC’s), and those that require neither Ca²⁺ nor DAG for activity (the ‘atypical’ aPKC’s) [58]. Diacylglycerols (DAG) are considered the classical lipidic activators of PKC. However, recent findings indicate that polyunsaturated fatty acids, either alone or in synergy with DAG, also act as modulators of the different PKC isoforms (Van Bilsen [59] and references herein, [60,61]). However, the significance of fatty acids as modulators of PKC activity in vivo still remains to be established. Furthermore, evidence has been obtained that the chemical nature of the phospholipid substrate may also be of importance. Musial et al. [62] showed that stimulation of mesangial cells with ET-1 led to the formation of DAG derived from ester-linked phospholipids, whereas in the case of interleukin-1α ether-linked phospholipids (plasmalogens) were preferentially used as substrate. These plasmalogen-derived DAG may act as inhibitors rather than activators of PKC-α [62].

Over the past couple of years various research groups have tried to establish which PKC isoforms are present in the different cell types within the cardiovascular system.
phenylephrine or ET-1 was associated with the activation of PKC-α, -δ, -ε, -ζ, and -η, whereas the presence of PKC-β could not be confirmed in all studies. In contrast to myocytes, PKC-β is abundantly present in vascular smooth muscle cells and endothelial cells and considered to be of special functional relevance under various pathophysiological conditions. For instance, activation of PKC-β has been implicated in the vascular pathology of diabetes [65,66].

It is generally assumed that the activation of distinct PKC’s results in the activation of different sets of downstream targets. The role of individual PKC isoforms in cardiovascular disease, with special emphasis on their mode of activation [67] and changes in their subcellular translocation in response to external stimuli [68], is still matter of intensive research. In this respect the role of so-called anchoring proteins, previously thought to be involved in protein kinase A signaling only, deserves attention [69]. These proteins assist in the sequestration of signaling units at discrete sites within the cell. Developmental changes in PKC isoform expression and/or activity have been reported by a number of groups [70–73]. In addition, it was recently shown that heart failure induced by a sequential volume- and pressure-overload was associated with reduced protein levels of PKC-α, -β1, -γ, and -ε, whereas the PKC-ζ remained unaltered [74]. Stimulation of neonatal cardiomyocytes with an α1-adrenergic agonist caused translocation of PKC-δ and PKC-ε, but not of PKC-α, from the soluble to membrane-associated fraction [67]. Likewise, stimulation of these cells with either phenylephrine or ET-1 was associated with the activation of different sets of PKC isoforms [75]. On the other hand, the observation that transfection of neonatal myocytes with either cPKC-α, nPKC-ε, or aPKC-ζ was equally effective in activating the ANF promoter [76] is not in favor of a divergent role of the distinct PKC isoforms.

Many studies, using either more or less specific synthetic activators (phorbolester or related analogues) or inhibitors (staurosporin, H7) have demonstrated the importance of PKC-mediated signaling in the regulation of processes as contractile protein function and (pathological) cell growth (see [52,77] for reviews). In view of the importance of the PKC-family it is actually surprising that relatively little is known about its target proteins. One of the major target proteins of PKC within the heart is the Myristoylated Alanine-Rich C-Kinase Substrate (MARCKS) [73]. The function of this ubiquitously expressed protein is largely unknown, although research on cancer has provided some indications that it may play a role in cell proliferation. It is generally accepted that PKC feeds into the mitogen activated protein kinase (MAPK, also referred to as ERK1/2) pathway, which acts as a convergence point for several signaling pathways.

A number of studies were designed to examine which PKC isoforms become activated upon Ang II stimulation. From the discussion above it will be apparent that this response is likely to be cell type specific. In COS cells stably transfected with the AT1 receptor the addition of Ang II led to the translocation of the α- and ε-isoforms, but not of the β-, γ-, or δ-isoforms of PKC [78]. Liao and coworkers [79] provided evidence that in cultured vascular smooth muscle cells the atypical PKC-ζ isozyme is responsible for the activation of MAPK by Ang II. Indeed transfection with PKC-ζ antisense oligonucleotides specifically downregulated PKC-ζ protein expression, and blunted the effect of Ang II on MAPK activation.

6.5. The PKC/MAPK connection

A likely candidate through which PKC might activate MAPK is the proto-oncogene c-Raf1, a component of the Ras–Raf–MAPKK–MAPK cascade (Fig. 2). c-Raf1 and its family members A-Raf and B-Raf act as MAPKK kinases and phosphorylate MAPKK on two serine residues. PKC has been shown to activate Raf via phosphorylation [80,81]. Mechanical stretch of neonatal cardiac myocytes led to the phosphorylation of c-Raf1 and MAPK in an Ang II and PKC-dependent manner, as both phosphorylation events were abrogated by pretreatment with either an AT1-receptor antagonist or a PKC inhibitor [82]. Endothelin-1 was also capable of transiently activating c-Raf1 and A-raf in neonatal cardiac myocytes [83]. Yamazaki and coworkers [84] reported that stimulation of neonatal cardiac myocytes with norepinephrine also resulted in Raf-1 and MAPK activation and that this effect is mediated by both the α1- and β-adrenergic receptor. Accordingly, a wide variety of hypertrophic agonists has been shown to activate Raf kinases. Together these findings indicate a central role of the Raf–MAPKK–MAPK pathway in signal transduction pathways associated with the development of cardiomyocyte hypertrophy, thereby further stressing the notion that MAPK forms a convergence point for various signaling cascades. The importance of MAPK is also illustrated by the fact that antisense oligonucleotides against MAPK attenuated the hypertrophic effect of the α1-adrenergic agonist phenylephrine on cardiomyocytes [85]. In addition transient transfection of these cells with expression vectors encoding dominant negative inhibitors of MAPK largely prevented the transcriptional activation of c-fos, ANF and MLC-2 promoter/luciferase constructs in phenylephrine-stimulated cells [86]. Nevertheless, various studies have also provided evidence that activation of the Raf-MAPK pathway may be required, but is not sufficient to explain all phenotypical alterations that are associated with cardiomyocyte hypertrophy [86–88]. Rho and Stress-Activated Protein Kinase dependent pathways (see below) are likely to play distinct roles in this process [89]. Interestingly the potential relevance of the MAPK pathway in the adaptation of skeletal muscle to physical exercise
Fig. 2. Schematic presentation which illustrates the coupling of the AT<sub>1</sub> receptor to downstream effectors, i.e., the p21 Ras →Raf →MAPKK →MAPK pathway, and the JAK/STAT pathway. The AT<sub>1</sub> receptor/G-protein coupled signaling cascades were already depicted in figure 1. Note the convergence of cytokine and Ang II signaling at the level of JAK, p21 Ras, and transcription factors. In addition the SAPK/JNK pathway is shown. For details and explanation of the abbreviations the reader is referred to the text. Activation of the AT<sub>1</sub> receptor ultimately results in a concerted general increase of mRNA and protein synthesis and the selective transcriptional activation of sets of genes through phosphorylation of a number of transcription factors.

was also demonstrated by the activation of Raf-1, MAPKK, and MAPK in exercising muscle [90].

Activation of MAPK has been shown to lead to the phosphorylation of various effector proteins, including cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>) [91], and the 90 kDa S6 kinase protein (p90<sup>rsk</sup>) [92] and the so-called ‘Phosphorylated Heat- and Acid-stable protein’ (PHAS1) [93]. In turn, p90<sup>rsk</sup> phosphorylates the S6 protein in the 40 S ribosome, whereas phosphorylation of PHAS1 results in the liberation of translation initiation factors complexed to this protein. Both events increase the efficiency of ribosomal protein synthesis. Furthermore, MAPK has been shown to phosphorylate, and thereby inhibit, Protein-Tyrosine Phosphatase 2C (PTP2C) [94]. By inference inhibition of PTP2C activity would prolong Tyrosine kinase-mediated signal transduction events that take place subsequent to Ang II stimulation. Finally, MAPK-dependent phosphorylation of transcription factors like c-jun and Elk-1 (p62<sup>Jun</sup>) has also been demonstrated [95,96]. Upon phosphorylation Elk-1 translocates from the cytoplasm to the nucleus, where it forms a ternary complex with the serum response factor (SRF) and the serum response element in the 5′-untranslated regions of several genes, among which the immediate early gene c-fos. The ternary complex enhances transcription of the c-fos gene [96].

In conclusion, stimulation of a wide variety of G-protein-coupled receptors that lack intrinsic tyrosine kinase activity, including the AT<sub>1</sub> receptor, indirectly leads to the activation of MAPK in a process involving tyrosine phosphorylation. The G-protein-coupled receptors share this property with the family of cytokine receptors. This notion has provided great impetus for the research into the role of cytokines in cardiovascular signal transduction and the relation between Ang II and cytokine-mediated signaling in recent years.

6.6. Signaling through p21 Ras

In view of the important role of Raf in signal transduction, the biological significance of p21 Ras, one of the activator proteins of Raf, in signal transduction in the cardiovascular system has also received widespread attention. Activated p21 Ras, a low molecular weight GTP-binding protein, recruits Raf to the cell membrane. In addition to phosphorylation, this is one of the ways by which Raf activity is regulated. Various hypertrophic agonists have been shown to activate p21 Ras [97]. Similar to the effects of G<sub>Gq</sub>, microinjection of constitutively active mutants of Ras induced ANF expression and neutralizing antibodies against Ras prevented ANF expression in cells following α-adrenergic stimulation [97].

The exact nature of the link between the G-protein-coupled receptors and p21 Ras has not been solved definitively. Current evidence indicates that the activation of p21 Ras is not the result of Gq activation, but rather occurs independently of Gq [51]. Recently it was demonstrated that in neonatal cardiac myocytes Ang II activates p21 Ras in a way analogous to the mechanism used by typical receptor tyrosine kinases like the platelet-derived growth factor (PDGF) receptor, i.e., via tyrosine phosphorylation of the linker protein Shc. This will lead to the subsequent association of Shc with the adaptor protein Grb2 (growth
factor receptor binding protein-2) and the guanine nucleotide exchange factor mSOS [98] (Fig. 2). Phosphorylation of the linker protein Shc in response to Ang II stimulation has also been observed in cardiac fibroblasts and smooth muscle cells [99,100]. The kinase responsible for the Ang II-induced tyrosine phosphorylation of Shc has not been identified with certainty, but members of the Src family, like Fyn, appear to be the most likely candidates [26,98].

Whatever the exact link may be, cardiac specific overexpression of p21 Ras in transgenic mice has been shown to be associated with ventricular hypertrophy [101]. However, this does not necessarily mean that under pathological conditions p21 Ras is critical for the development of cardiac hypertrophy. Recent studies by Abdellatif and coworkers [102] suggest that p21 Ras has a global effect on gene expression, rather than being responsible for the induction of the fetal gene program, which is considered a hallmark of the hypertrophic phenotype. Recent data suggest that this general effect on global gene expression is mediated by the phosphorylation of RNA polymerase II (Fig. 2). In fact, there is an ongoing discussion as to the relative importance of PKC, Src, and p21 Ras as activators of the Raf–MAPK pathway [98,103,104].

6.7. The JAK/STAT pathway

As indicated before there are obvious similarities between the signaling through G-protein-coupled receptors and cytokine receptors. The binding of interferons and interleukins to their cognate receptors results in receptor dimerization and autophosphorylation and activation of the associated Janus Kinases (JAKs) [105]. So far various members of this family of tyrosine kinases have been identified (JAK1, JAK2, Tyk2, JAK3). Recent studies have shown that, similar to cytokine receptor activation, activation of the AT1-receptor results in activation of JAK2 [106]. Conclusive evidence for a direct interaction between the AT1 receptor and JAK2 was provided by the co-immunoprecipitation of these two proteins. Activation of JAKs will initiate recruitment of other proteins to the activated receptor complex resulting in their phosphorylation on tyrosine residues. Important substrates are the members of the STAT (Signal Transducer and Activator of Transcription) family of proteins [107]. The STAT proteins contain SH2 and SH3 domains important for protein–protein interaction. Upon tyrosine phosphorylation the STAT proteins dimerize. As homo- or hetero-dimers they are translocated to the nucleus and are able to bind to consensus DNA sequences often referred to as Interferon-Stimulated Response Elements (ISRE). Indeed, activation of the AT1 receptor resulted in the phosphorylation of JAK and STAT in vascular smooth muscle cells [106]. Subsequent studies showed that the AT1-JAK-STAT cascade was also operative in neonatal cardiac fibroblasts [108]. Furthermore, recent studies [109,110] provided evidence for extensive cross-talk between Ang II and cytokine-mediated signaling at the level of MAPK and STAT phosphorylation. As discussed in more detail below the JAK–STAT pathway is also operative in cardiomyocytes.

7. Signaling through cytokine receptors

One of the most exciting developments in the research on cardiovascular signaling concerns the role of cytokines as paracrine factors and the elucidation of cytokine receptor function and its downstream signaling pathways (Ref. [111] for review). The potential significance of the production of pro-inflammatory cytokines in cardiovascular diseases [112,113] and their effects on cardiac muscle function [114] has been appreciated for a number of years. However, an important milestone in realizing that cytokines play an important role in cardiac signaling came from the observation that conditioned medium from embryoid bodies contained a factor that acted as a potent stimulus for the induction of hypertrophy in neonatal cardiac myocytes [115]. Expression cloning strategies led to the identification of a protein, referred to as Cardiotrophin 1 (CT-1), the amino acid sequence of which is highly homologous to interleukins (IL) and Leukemia Inhibitory Factor (LIF).

Signaling through cytokine receptors involves gp130, which acts as a signal-transducing receptor component (Fig. 2). Binding of ligands to the cytokine receptors results in the formation of receptor complexes, of which gp130 forms an essential part. Gp130 itself possesses no intrinsic tyrosine kinase activity, but following its dimerization with cytokine receptors associated tyrosine kinases, like the JAKs, gp130 becomes activated. In turn, the JAKs will phosphorylate and activate their substrates, members of the STAT family [111]. Interestingly a number of studies indicate that the Ras–Raf–MAPKK–MAPK pathway is also activated following cytokine receptor activation [116,117]. The activation of this pathway through cytokine receptors also involves gp130, but the nature of the link between gp130 and Ras is less well understood. Using transgenic and knockout technologies the importance of gp130 in cardiac growth has been demonstrated. Knockout mice that are deficient in gp130 display a hypoplastic development of the ventricular compartment, resulting in premature death [118]. Conversely, in mice overexpressing IL-6 as well as its receptor, gp130 is continuously active. These double transgenic mice a characterized by a marked hypertrophy of the heart [119].

Administration of CT-1 to neonatal cardiac myocytes resulted in cardiomyocyte hypertrophy [120,121]. Similar findings were observed when LIF was applied to these cells [122]. It should be noted that at the gene level CT-1 induced a different kind of hypertrophic phenotype than that elicited by ‘classical’ hypertrophic stimuli, such as α-adrenergic agonists, ET-1, or Ang II. Stimulation with
cytokines did not result in an enhanced expression of skeletal α-actin, β-myosin heavy chain (βMHC) or the ventricular isoform of myosin light chain-2, but induction of expression of the hypertrophic marker ANF still occurred. Moreover, IL-1β was found to attenuate upregulation of βMHC expression as induced by the α1-adrenergic agonist phenylephrine [123].

CT-1 signaling in neonatal cardiomyocytes has been shown to involve the heterodimerization of gp130 and the LIF-receptor [121,124], which leads to the activation of the JAK/STAT pathway and of the MAPK pathway. Recent findings strongly suggest that each of these two pathways might be involved in different processes. Sheng and coworkers [117] provided evidence that CT-1 mediated activation of the JAK/STAT pathway is most likely involved in the hypertrophic response, whereas CT-1 mediated MAPK activation inhibits apoptosis of cardiomyocytes. Together, these recent findings have provided important clues as to the importance of cytokine-mediated signaling in the cardiovascular system.

8. Stress-activated protein kinases

Recently a new group of serine/threonine kinases has been identified [125]. These kinases are related to MAPK and are referred to as Stress-Activated Protein Kinases (SAPKs) or alternatively, c-Jun N-terminal kinases (JNKs) as the immediate early gene c-Jun was the first substrate identified. The phosphorylation of the transactivation domain of c-jun results in the activation of the transcription factor complex AP-1, a fos/jun dimer, thereby increasing the rate of transcription of genes containing AP-1 responsive elements in their untranslated regions. As the name implies SAPK becomes activated in response to stress-inducing conditions, like ischemia/reperfusion and heat shock (see [126] for review). The activation cascade of SAPK involves two upstream threonine/serine kinases referred to as MEKK-1 and SEK-1 (Fig. 2), analogous to the MAPKK (MEK)–MAPKK (MEK)–MAPK (ERK) module [127]. However, so far it is incompletely understood how the stress-signals are relayed to the MEKK-1–SEK-1–SAPK pathway. There are indications that the activation of SAPK/JNK via G-protein-coupled receptors may involve the βγ-subunit of the heterotrimeric G-proteins [128].

Using cultured neonatal cardiomyocytes as a model system it was shown that the reoxygenation event, rather than the preceding hypoxic episode, provides the stimulus for SAPK/JNK activation [129]. Comparable observations were made with isolated rat hearts subjected to ischemia and reperfusion [130]. Circumstantial evidence indicated that reactive oxygen species, generated during reoxygenation were involved in this response. It has also been shown that the imposition of an osmotic stress on cardiomyocytes activates SAPK/JNK [131]. This observation fits with the idea that transcellular fluid shifts, associated with reperfusion of previously ischemic tissue, provide a trigger for SAPK/JNK activation. Recently, it was shown that Ang II administration to neonatal cardiomyocytes resulted in a rapid activation of SAPK/JNK in a manner that involved the AT1 receptor and required both PKC activity and elevation of intracellular Ca2+ levels [132]. Mechanical stretch was also found to activate SAPK/JNK, but unlike the activation of MAPK, this response appeared to be Ang II-independent [133]. Furthermore, α1-adrenergic stimulation was also associated with increased SAPK/JNK activity. In this case the activation of SAPK/JNK appeared to be p21 Ras dependent [134]. Recently it was observed that the lipid second messenger ceramide also mediated SAPK/JNK activation [135]. The role of ceramide in signal transduction is potentially of great importance as it has been shown to modulate a variety of cellular responses, including cell differentiation and apoptosis [136,137]. Although only recently discovered, the assembled data already indicate that the SAPK/JNK pathway may be very relevant to the adaptation of the cardiovascular system in response to disturbances in cardiovascular homeostasis.

9. Conclusions

Since the publication of the spotlight issue in this journal in the fall of 1995 a number of exciting new developments have taken place, only a selection of which have been highlighted. This review mainly focused on the role of Ang II mediated signaling pathways as the deciphering of the downstream signaling cascades has progressed a lot over the last years. Accordingly, Ang II-mediated signaling provided a good example to illustrate the complexity of the downstream signaling cascades, with respect to both the number of cascades involved and their interrelationships. However, this does not imply that other peptide growth factors are of less relevance. Signaling through the endothelin-1 and Insulin-like growth factor 1 (IGF-1) receptors will probably prove to be of equal biological importance for the cardiovascular system [138]. In fact the potential beneficial effects of the growth hormone–IGF-1 axis in relieving cardiac failure [139], and the therapeutic effects of endothelin-1 receptor antagonists [140], are currently receiving widespread attention in cardiac research.

The ongoing development of new cell biological and molecular techniques will further accelerate the elucidation of components of signaling cascades and the mutual interactions between signaling pathways. In this respect the recent development of new cloning techniques based on protein-protein interaction will be fruitful [141]. Furthermore, especially research on cardiac cell signaling has been hampered by the inability to use isolated adult cardiomyocytes as a model system as a result of which most
studies concerning signal transduction during the hypertrophic response have been performed with neonatal myocytes. Although important concepts and clues have been (and are still being) obtained with this model system, extrapolation of findings from the neonatal to the adult phenotype is not without risk. However, the establishment of culture conditions (composition of the medium, pacing) that allow isolated adult myocytes to maintain their differentiated phenotype for extended periods of time [142,143], in combination with Adenoviral transfection techniques that result in a nearly 100% transfection efficiency of neonatal as well as adult myocytes [144], will bridge this gap in the years to come. Finally, transgene and gene knock-out strategies in mice have already provided important information about the biological significance of particular proteins involved in signal transduction, and about counter-regulatory mechanisms that are operative to maintain cardiovascular homeostasis. The advent of physiological techniques that allow in vivo and ex vivo manipulation and/or functional analysis of the murine cardiovascular system is likely to fuel future research into the biological significance of signal transduction pathways in the vessel wall and heart, and will allow the testing of leads obtained with cellular model systems in an integrated system.

In view of the substantial progress that has already been made within the last couple of years, it will be interesting to see to what extent the black box of cardiovascular signal transduction has been opened at the start of the next millennium.

10. List of abbreviations

ACE Angiotensin Converting Enzyme
Ang II Angiotensin II
AP-1 Activator Protein-1 (fos/jun dimer)
AT₁ Angiotensin II receptor Type 1
AT₂ Angiotensin II receptor Type 2
ANF Atrial Natriuretic Factor
Cdk Cyclin dependent kinase
CT-1 Cardiotrophin-1
Elk-1 transcription factor (= p62TCF)
ERK-1 Extracellular-signal related kinase 1 (= p44)
ERK-2 Extracellular-signal related kinase 2 (= p42)
ET-1 Endothelin-1
FAK Focal Adhesion Kinase
GAP GTPase -activating protein
G-protein GTP-binding protein
Grb2 Growth factor receptor binding protein 2
IFN Interferon
IL Interleukin
ISRE Interferon-Stimulated Response Element
JaK Janus Kinase
JNK c-Jun N-terminal kinase (= SAPK)

LIF Leukemia Inhibitory Factor
MAPKK Mitogen Activated Protein Kinase Kinase (= MEK)
MAPK Mitogen Activated Protein Kinase (= ERK)
MARCKS Myristoylated Alanine-Rich C-Kinase Substrate
mSOS guanine nucleotide exchange factor, mammalian form of drosophila Son of Sevenless
PHAS1 Phosphorylated Heat- and Acid-Stable protein 1
PKC Protein Kinase C
PLC phospholipase C
PTP2C Protein-Tyrosine Phosphatase 2C
p90RSK ribosomal S6 kinase
SAPK Stress-Activated Protein Kinase
SH2 Src-homology domain 2 (src = Rous Sarcoma virus oncogene)
STAT Signal Transducer and Activator of Transcription

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