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OUABAIN-INDUCED CELL SIGNALING

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1. ABSTRACT

When endogenous ouabain was first isolated from human plasma in 1991, many expressed doubts that such a compound could be endogenous to humans because of its unusual structure, its unknown synthesis, and its unidentified site of origin. Furthermore, the relevance of human ouabain was questioned because of its apparently low (≤nmol/L) circulating concentration. Since then, much progress has been made on the origin and synthesis of endogenous ouabain, but perhaps the most significant finding is that nanomolar concentrations of ouabain can induce numerous signal transduction events in both primary and immortalized cultures of cells. Here we analyze the effects of low ouabain-induced signals including cell proliferation, calcium mobilization, cell cytotoxicity, apoptosis, mitogen-activated protein kinase (MAPK) activation and other signaling pathways. Furthermore, we consider how these low dose ouabain-induced events might enable a putative role for human endogenous ouabain to be assigned.

2. INTRODUCTION

In 1991, John Hamlyn and colleagues from the University of Maryland, in collaboration with a group from the Upjohn Laboratories, Kalamazoo, produced several anuscripts that suggested that the cardiac glycoside ouabain (or an isomer), was endogenous in humans (1-3). This was a challenging finding for many reasons but most principally because (a) the structure of ouabain is unlike that of any other known endogenous steroid and (b) the presence of such low concentrations of circulating ouabain (or ouabain-like compound) in plasma was considered by many to be physiologically irrelevant. Furthermore, there was much controversy over the site of where endogenous ouabain was produced. In recent years our understanding of how ouabain and related cardiac glycosides are synthesized (4,5), and where they originate have improved significantly (5,6), but perhaps the greatest leap in our knowledge has been the discovery that ouabain can influence cell signaling pathways via the Na^+K^+-ATPase, without necessarily affecting Na^+ and K^+ gradients. Whilst some distinguished observers still question the significance of endogenous ouabain (7), this has been largely overshadowed by the recent explosion in the number of papers that demonstrate diverse effects of nanomolar concentrations of ouabain on both primary and immortalized cells (8-16).

Here, we review the recent discovery that low concentrations of ouabain can be a potent activator of numerous cellular processes and assess the possible

implication of these findings for our understanding of the role of endogenous ouabain in human physiology and pathophysiology.

3. OUABAIN AND THE NA^+K^+-ATPASE

High concentrations of ouabain (≥μM) are toxic and this was originally thought to be due to the classical inhibition of the Na^+K^+-ATPase causing a rise in intracellular Na^+, which produces cell injury by two pathways. Firstly, the accumulation of intracellular Na^+ can lead to the eventual dissipation of the electrochemical plasma membrane potential which is accompanied by a buildup of inorganic ions, causing cell swelling and necrotic death. Secondly, the rise in intracellular Na^+ causes the activation of the Na^+/Ca^{2+} exchanger which eventually leads to a sustained rise in intracellular Ca^{2+}, which in turn mediates cell injury/death. The inotropic effect of digoxin, ouabain, and other related cardiac glycosides are thought to occur by transforming the rise in intracellular Ca^{2+} into an increase in force of the contracting myocardium. However, recent studies have indicated that the Na^+K^+-ATPase can initiate signal transduction pathways at ouabain concentrations that are too low to induce a rise in intracellular Na^+ (8-11, 17). Furthermore, ouabain concentrations that are known to significantly inhibit Na^+K^+-ATPase ion pump function are now thought to be able to cause injury by non-ionic events (18-19), to cause the generation of a reactive oxygen species (ROS, 15, 17), and to initiate numerous protein kinase cascades (20), Lastly, evidence that nanomolar concentrations of ouabain can actually stimulate Na^+K^+-ATPase activity has been known for more than 30 years, and has been demonstrated by many workers during this time (11, 16, 21-25). However, the reason for this and its possible implications are unclear but it supports the suggestion that the Na^+K^+-ATPase can function in a non-classical manner.

4. SIGNAL TRANSDUCTION EVENTS INDUCED BY NANOMOLAR CONCENTRATIONS OF OUABAIN

The recent discovery that low concentrations of ouabain (and other cardiac glycosides) can elicit signal transduction events via the Na^+K^+-ATPase that are unrelated to changes in Na^+/K^+ gradients has opened the possibility of assigning human endogenous ouabain with physiological and pathophysiological functions. Evidence suggests that nanomolar concentrations of ouabain induce cell proliferation (8-11, 16), calcium mobilization (12-13, 15-16), cell cytotoxicity (9, 15), protease activation (9, 14), and MAPK phosphorylation (9, 11, 16). These events and other examples of low-ouabain-induced signal transduction will be discussed further in this review.

4.1 Cell proliferation

Primary vascular smooth muscle cells from human and canine sources have been shown to proliferate maximally when treated with 1 nmol/L ouabain (8, 10). Maximal growth stimulation at the same concentration of ouabain was also found in primary rat renal epithelial cells (11), whilst 5 nmol/L of ouabain induced the greatest degree of proliferation in human umbilical cord endothelial cells (HUAEC, 16). Human primary prostatic smooth muscle cells were found to proliferate maximally at even lower concentration of ouabain (0.1 nmol/L, 9). Ouabain is thought to induce its proliferative (and hypertrophic) effect by activating the Ras/Raf/MEK/ERK cascade (20, see *MAPK signaling*).

In all of these studies ouabain was shown to stimulate increasing cell proliferation until reaching a certain peak concentration, from which higher concentrations of ouabain produced lower proliferative rates. It can be speculated that endogenous ouabain might therefore exert control on the development of certain tissues which is then arrested when ouabain falls below, or goes above, a narrow concentration window.

4.2. Calcium mobilization

Calcium is a highly versatile intracellular signal that regulates a vast number of cellular functions. The calcium signal is derived from either internal stores or from the extracellular medium. Calcium homeostasis is regulated by an array of receptors, transducers, ion channels, buffers, effectors, ion pumps and exchangers. Originally it was thought that ouabain could affect intracellular calcium concentrations by direct inhibition of the Na^+K^+-ATPase, causing a rise in intracellular Na^+ followed by a rise in intracellular Ca^{2+} via the activation of the plasma membrane bound Na^+/Ca^{2+} exchanger. However, intracellular Ca^{2+} is known to rise in response to ouabain in the absence of a rise in intracellular Na^+ (26). This has lead to speculation that ouabain-induced elevations in intracellular Ca^{2+} might be due to signal transduction events rather than as a sole consequence of changes in cell Na^+/K^+ ratios. This seems especially likely when considering examples of nanomolar ouabain-induced rises in intracellular calcium (16, 27). The rise in Ca^{2+} has been shown to result from a sharp increase in calcium influx from the extracellular media in prostate cancer (PC-3) cells (15). This low ouabain-induced Ca^{2+} rise has been further supported by the observation that the chelation of extracellular Ca^{2+} could prevent the low dose ouabain-induced CD69 expression in murine thymocytes (28). Furthermore, ouabain-induced Ca^{2+} oscillations in primary renal proximal tubule cells could be halted by incubating the cells in a Ca^{2+} free buffer (12). Similar low frequency Ca^{2+} oscillations were found in HUAECs incubated with 1 nmol/L ouabain (16).

The eventual outcome of low dose ouabain-induced elevated, or oscillating, intracellular Ca^{2+} is expected to be diverse. Many of the observed effects of nanomolar ouabain treatment, such as loss of mitochondrial membrane potential (15), production of ROS (15), protease activation (9, 14), apoptosis (9, 14, 28-29), NFκB activation (12), endothelin release (16) and MAPK activation (8, 9, 11, 16), have been shown to be either directly, or indirectly, as a result of an increase in intracellular Ca^{2+} (9, 12, 14, 16, 28). These data, in conjunction with the known proliferative effect of low dose ouabain, suggests that endogenous ouabain might play a

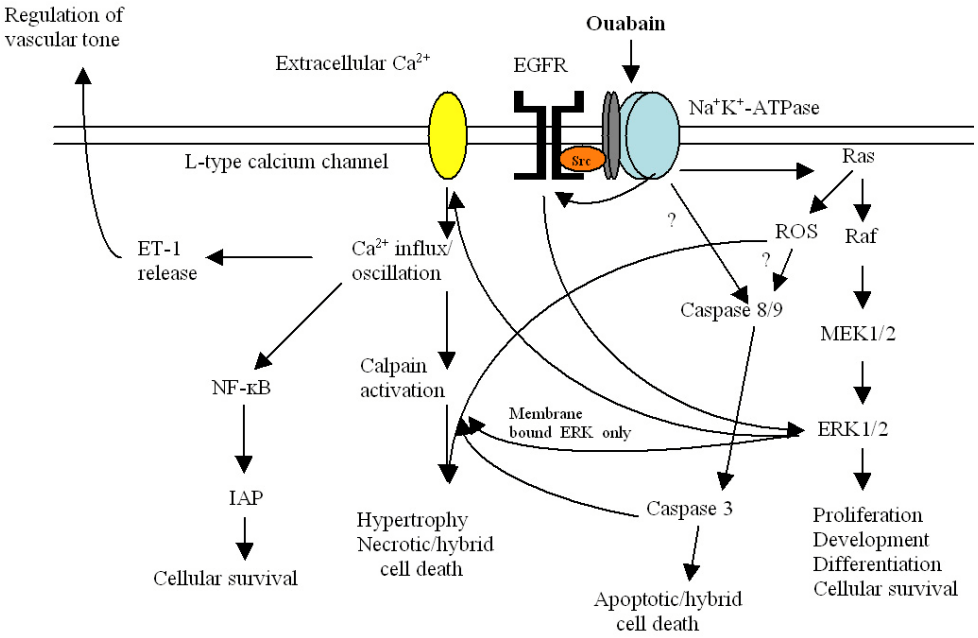

Figure 1. Simple schematic representation of some of the signaling events initiated by ouabain (excluding ionic effects due to changes in the intracellular Na^+/K^+ ratio). The majority of the pathways shown here have been demonstrated using nanomolar ouabain concentrations upon primary and immortalized mammalian cells in culture (see text for details). EGFR, epithelial growth factor receptor; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ET-1, endothelin-1.

role in cell homeostasis because cell growth (hypertrophy and proliferation), cell death and survival pathways (e.g. via NF-κB, see *Other ouabain-induced effects*) can all be initiated by ouabain.

4.3. Cell cytotoxicity and protease activation

Despite the fact that ouabain has been used extensively in life science research since it was first crystallized in 1888 (30), it has only been recently recognized that nanomolar concentrations can be cytotoxic (9, 15). In human prostatic smooth muscle cells, 10 nmol/L ouabain produced a significant rise in both LDH release and in the activity of the cysteine protease caspase-3 (9). In human prostate adenocarcinoma PC-3 cells, 3 nmol/L ouabain was found to cause a modest reduction in mitochondrial membrane potential (15). In the same cell line, related cardiac glycosides such as oleandrin and digoxin were shown to induce apoptosis (29). Furthermore, higher concentrations of ouabain (100 nmol/L) can also induce apoptosis (TUNEL reaction), which can be greatly attenuated by caspase-3 inhibition (9).

Low doses of ouabain (10 nmol/L) are known to activate the calcium-dependent cysteine protease calpain in human derived myoblastic cells (Girardi). Calpain is a mediator of cell death and its elevation in activity in response to ouabain appears to be mediated by Ca^{2+} influx (14). Recently, we studied calpain activity in four distinct cell cultures: Girardi (derived from human heart), LLC-PK_1 (pig kidney proximal tubule), HK-2 (human kidney proximal tubule) and rat primary vascular smooth muscle cells (VSMC). We found that calpain was significantly activated by nanomolar concentrations of ouabain within 3 h in all the cell cultures investigated (31). This finding fits well with recent work which showed that transactivation of the epidermal growth factor receptor (EGFR) occurred in canine VSMCs treated with 1 nmol/L ouabain (8). This resulted in ERK phosphorylation, which has been shown to be a prerequisite for calpain 2 (m-calpain) activation (32-34).

Collectively, these data suggest that low doses of ouabain (nanomolar) can be cytotoxic and are able to activate cell death effectors such as caspase-3 and calpain, proteases that are known to crosstalk (35). Calpain activation can lead to apoptosis, necrosis, and the induction of calcium-triggered cell death via the cleavage of endogenous calcineurin inhibitor cain/cabin1 (36). Whilst higher concentrations of ouabain have been shown to be both anti- (37-39) and pro-apoptotic (9,15,19,40) it seems that low dose ouabain is an inducer of cell death (9,15, 29). Ouabain-induced cell death (low or high concentrations) appears to occur through a combination of both necrotic and apoptotic pathways (9), which has been referred to as hybrid cell death (41). Factors such as cell type, ouabain concentration, time of ouabain exposure, and the effect of additional cellular insults (such as irradiation) need to be considered before defining a specific role of endogenous ouabain in cell death. However, enough evidence has accrued recently concerning the effects of nanomolar concentrations of ouabain in multiple cell lines to propose that endogenous ouabain might be involved in cell death cascades and other signaling events (Figure 1).

4.4. MAPK signaling

The mitogen-activated protein kinase (MAPK) superfamily consists of three signaling pathways: the extracellular signal-regulated protein kinases (ERKs), the

c-Jun N-terminal kinase or stress activated kinases (JNK/SAPK), and the p38 family of kinases (42). Nanomolar concentrations of ouabain have been shown to activate growth-related and cell survival pathways, leading to the phosphorylation of p42/44 MAPKs (8, 9, 11, 16), also known as ERK1 and ERK2. Furthermore, rats treated with ouabain were found to have a higher basal level of heart ERK1 and ERK2 phosphorylation than that found in untreated animals (43). Using relatively high ouabain concentrations (10-100 μmol/L) on rat cardiac myocytes (44), the sequence of the signal progression was determined. Ouabain was shown to rapidly activate the protein Ras (within 15 min) via the tyrosine kinase Src. This then activates Raf, which in turn activates MEK, which causes the phosphorylation ERK1 and ERK2. In addition, 1 nmol/L ouabain was found to transactivate EGFR causing ERK phosphorylation in canine VSMC (8). There is evidence that ouabain-induced signaling cascades are sometimes associated with the production of ROS, which is thought to play a role in the development of hypertrophy (20, 45-46) and apoptosis (47-48, see Figure 1).

5. OTHER OUABAIN-INDUCED EFFECTS

5.1. NF-κB

NF-κB is a transcription factor that modulates the activity of genes during the immune response and in cell proliferation and apoptosis (49). NF-κB activation is able to reduce apoptosis by upregulating inhibitors of apoptosis (IAP), which can restrict caspase activity (50). Recent work has revealed that NF-κB can be activated by ouabain in primary cultures of rat cardiac myocytes (51) and kidney proximal tubular cells (12). Moreover, the activation of NF-κB could not be achieved by inhibiting Na^+K^+-ATPase activity by a lowering in extracellular K^+, a process that does not elevate intracellular Ca^{2+}, which suggests that ouabain activation of NF-κB is a Ca^{2+}-dependent event, probably mediated via by L-type voltage channels (12). Further work has demonstrated that the physical association of the inositol 1.4.5-trisphosphate receptor with Na^+K^+-ATPase is essential for the generation of Ca^{2+} oscillations that are able to initiate NF-κB activation (13).

5.2. Endothelin-1 release

Endothelin-1 (ET-1) is a potent vasoconstrictor and promoter of cell growth in VSMCs and is known to inhibit the activity of the Na^+K^+-ATPase. ET-1, has been shown to activate ERK1 and ERK2 in perfused rat hearts (43). Rats that had been pre-treated with ouabain produced a larger increase in ERK phosphorylation than control animals (43). Other work suggests that 1 nmol/L concentrations of ouabain is able to induce the release of ET-1 within minutes of treatment on primary cultures of HUAECs (16). This might be the mechanism by which endogenous ouabain can exert vascular effects and possibly induce the pathogenesis of essential hypertension.

6. DISCUSSION

The finding that ouabain is able to induce signal transduction events has reactivated debate as to the physiological and pathophysiological role of endogenous ouabain (7, 51). The number of articles showing that nanomolar (and sub-nanomolar) concentrations of ouabain can elicit cell proliferation (8-11,16), activate cell death (9,14,28) and promote cell survival pathways (8-12,16) adds further fuel to the argument that endogenous ouabain plays an active role in mammalian biology. It is interesting to note that the concentration of exogenous ouabain (100 pmol/L) required to cause proliferation *in vitro* (8) is the same as that found *in vivo* in the plasma of healthy human volunteers (52). In addition, it should be remembered that other endogenous cardiac glycosides have been identified in mammalian plasma. These include ouabain-related cardenolides such as digoxin-like compound (5), and bufadienolides such as marinobufagenin (53) and proscillaridin A (54). It seems plausible that the cumulative concentration of all these factors (including endogenous ouabain) would be sufficient to induce multiple signaling events in humans.

However, whilst it seems entirely feasible that endogenous ouabain can exert an influence physiologically in humans, it is still unknown how it might act (with other cardiac glycosides) in an ordered and regulated manner like the other known human hormones. Some control of ouabain signaling might be attributed to the distribution of Na^+K^+-ATPase complexes that contain ouabain sensitive α-subunits that are juxtaposed to sarcoplasmic or endoplasmic reticulum into plasma membrane bound microdomains (termed PLasmERosomes see 26, 55-56). These complexes are thought able to influence calcium signaling via the Na^+/Ca^+ exchanger at nanomolar concentrations of ouabain. Another possible mechanism of control might be achieved by a restriction on the number of Na^+K^+-ATPase that are closely associated with the proteins (such as EGFR and Src) that are required to transmit ouabain signals (20). In addition, it appears likely that ouabain might act upon a specific tissue in a concentration-dependent manner. At sub-nanomolar ouabain concentrations it might cause cell proliferation (8,11), whilst at higher concentrations where ouabain-induced proliferation is more restricted it could induce apoptosis (9), thereby controlling tissue growth.

The tissue proliferation and hypertrophy found in sub-totally nephrectomized rats in kidneys (11) and heart left ventricles (57) could be initiated by elevated endogenous ouabain concentrations which are known to occur in uremia (58). This is supported by the evidence that raised ouabain concentrations are associated with the development of left ventricular hypertrophy (LVH) in hypertensive patients, and with cell hypertrophy in experimental models (59-60). Therefore, it can be speculated that essential hypertension, hypertension associated with chronic renal failure and the progression of LVH, might, in part at least, be due to elevated plasma ouabain concentrations. Ouabain activation of calpain might be an initiator of hypertrophy (14), and calpain activity is known to be elevated in the uremic rat myocardium (57). Ouabain-induced activation of ET-1 (and possibly vascular cell proliferation) could be the reason for the increase in vascular tone and the raising of blood pressure in the uremic population (16).

In conclusion, recently published articles demonstrating that nanomolar concentrations of ouabain can initiate numerous signal transduction events has caused a re-evaluation of the potential function of human endogenous ouabain. Whilst the precise physiological and pathophysiological role of endogenous ouabain is still far from being resolved, recent evidence suggests that we are at last getting closer to some understanding of how this intriguing and unusual steroid might function in human biology.

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