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REVIEW ARTICLE

Role of Calcium in Glucocorticosteroid-Induced Apoptosis of Thymocytes and Lymphoma Cells: Resurrection of Old Theories by New Findings

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MORE THAN 50 years ago, investigators observed that the thymus gland undergoes hypertrophy in association with adrenocortical insufficiency and atrophy in association with adrenocortical excess.[1,2] These observations led to the realization that adrenal corticosteroids (ACS) have a ''lympholytic effect,'' which in turn stimulated clinical investigators to treat lymphomas with ACS for the first time in the late 1940s.[3,4] Today, we know that ACS do not directly lyse thymocytes and lymphoma cells, but rather induce apoptosis.[5] Apoptosis, or programmed cell death, is a genetically regulated process in which the cell is active in producing its own death, a type of cellular suicide for the sake of maintaining homeostasis in the cellular community.[6-8]

Although our understanding of apoptosis has advanced tremendously in recent years, the mechanism by which ACS induce apoptosis in thymocytes and lymphoma cells is not completely understood. In the 1970s, Sibley and Tompkins determined that the initial step in ACS-induced apoptosis is mediated through the ACS receptor and requires translocation of the receptor from the cytoplasm into the nucleus.[9] In the nucleus, the ACS receptor functions as a transcription factor, enhancing or repressing the expression of a selected repertoire of genes.[10] ACS may repress expression of genes necessary for cell survival by attenuating AP-1 (c-Fos/c-Jun) transcription factor activity,[11] or may induce the transcription of genes involved in carrying out the death program. The evidence in support of the latter concept is twofold: first, inhibitors of RNA and protein synthesis inhibit ACS-induced apoptosis[12,13]; and second, the transactivation domain of the ACS receptor is required for apoptosis induction by ACS.[14]

Progress in identifying ACS-inducible genes that mediate apoptosis had been slow. Now, in the past year, two ACS-inducible genes have been formally implicated in mediating apoptosis. One of the genes encodes a purinergic receptor, $P2X_1$, that functions as an ATP-gated calcium channel,[15] whereas the other gene encodes an inositol 1,4,5-trisphosphate receptor (IP_3R) that functions as an IP_3-gated calcium channel.[16] The fact that both of these genes encode calcium channels has reawakened interest in the role of calcium, a major intracellular second messenger molecule, as a mediator of apoptosis. In this review, we first summarize the evidence for involvement of these genes in apoptosis. Then, we attempt to integrate these novel findings with previous experimental evidence suggesting a role for calcium in signaling glucocorticoid-induced apoptosis in both thymocytes and lymphoma cells.

$P2X_1$ RECEPTORS AND APOPTOSIS

Recognition that purinergic receptors are involved in ACS-induced apoptosis evolved out of an effort by Owens et al[17] to identify differentially expressed mRNAs associated with ACS-induced apoptosis in thymocytes. One of the differentially expressed mRNAs identified by these investigators, termed RP-2, was induced early in the course of ACS-induced apoptosis. However, the identity of the RP-2 sequence was not elucidated until 5 years later, when it was discovered that RP-2 corresponds to a partial sequence of a gene encoding an ATP-gated cation channel, termed purinergic receptor or P_{2X} receptor.[18,19] This discovery is particularly intriguing, because extracellular ATP has been reported to induce apoptosis in thymocytes by increasing the intracellular concentration of calcium.[20,21]

The P_{2X} receptors constitute a family of at least seven members, distributed throughout central and peripheral neurons, smooth muscles, epithelia, developing skeletal neuromuscular junctions, lymphocytes, platelets, and macrophages. P_{2X} receptors are nonselective cation channels with significant permeability to calcium. P_{2X} receptors engage in intercellular communication by detecting the regulated (synaptic) or lytic (cell death) release of intracellular metabolites such as ATP. Unlike other well-known ion channels gated by extracellular ligands (eg, nicotinic, serotonin, $5\text{-}HT_3$), P_{2X} receptors are characterized by only two transmembrane domains with intracellular amino- and carboxy-termini.[22] RP-2 corresponds to the subfamily member $P2X_1$ (originally termed $P_{2X}R1$), recently cloned from vas deferens and PC12 cells.[18,19] Curiously, P_{2X} receptors are structurally similar to some of the *Caenorhabditis elegans* gene products implicated in neuronal cell selection and death.

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The link between $P2X_1$ receptor expression and ACS-induced apoptosis in rat thymocytes was recently established by Chvatchko et al.[15] These investigators found that $P2X_1$ receptors were upregulated in thymocytes during ACS-induced apoptosis. Furthermore, extracellular ATP enhanced ACS-induced apoptosis and antagonists of ATP substantially reduced ACS-induced apoptosis, suggesting that ACS-induced apoptosis is dependent on $P2X_1$ receptor activation by extracellular ATP.

IP_3R AND APOPTOSIS

The other ACS-inducible gene implicated in mediating apoptosis of both thymocytes and lymphoma cells encodes the Type 3 IP_3R.[16] The IP_3R has been extensively characterized and plays a central role in calcium signaling.[23,24] Typically, IP_3 generated in response to G-protein–coupled receptors and receptor tyrosine kinases binds to the IP_3 receptor that spans the endoplasmic reticulum (ER) membrane.[23,24] The binding of IP_3 induces transient opening of the receptor, allowing calcium to flow from the ER lumen into the cytoplasm, thereby producing a transient elevation of cytosolic calcium that in turn activates signal transduction kinases. Although IP_3R are most prominently located on the ER membrane, there is evidence for localization of the IP_3R to the plasma membrane, enabling the IP_3R to mediate entry of extracellular calcium into the cytoplasm[16] (and references therein). In cells undergoing apoptosis in response to ACS, immunocytochemical studies localized the IP_3R (specifically subtype 3) to the plasma membrane.[16] Upregulation of IP_3R in S49 lymphoma cells was associated with increased cytosolic calcium concentration, suggesting that increased expression of IP_3R on the plasma membrane increased calcium entry.[16] Significantly, repression of ACS-induced IP_3R expression by transfecting cells with an IP_3R antisense plasmid not only decreased the induction of IP_3R, but also inhibited the induction of apoptosis by ACS.[16]

RELATIONSHIP OF $P2X_1$ AND IP_3R TO EARLIER EVIDENCE LINKING CALCIUM WITH ACS-INDUCED APOPTOSIS

The involvement of calcium in ACS-induced apoptosis was first suggested by Kaiser and Edelman,[25] who discovered that extracellular calcium is necessary for induction of apoptosis in thymocytes by ACS. This observation, which has been confirmed by others,[12,13] suggests that extracellular calcium uptake mediates ACS-induced apoptosis. The concept that calcium signals ACS-induced apoptosis is further supported by evidence that the calmodulin inhibitor, calmidazolium, interferes with ACS-induced thymocyte apoptosis.[26,27] However, in contrast to the situation in thymocytes, extracellular calcium is unnecessary for ACS-induced apoptosis of peripheral lymph node lymphocytes and lymphoma cells.[28-33]

These earlier findings are fully consistent with the patterns of expression of the $P2X_1$ receptor. $P2X_1$ expression was detected in thymocytes, but not in peripheral (lymph node) T lymphocytes.[15] Furthermore, there is a strong correlation between $P2X_1$ expression and the susceptibility of individual thymocyte subsets to ACS-induced apoptosis. In the thymus gland, immature $CD4^+CD8^+$ thymocytes residing within the cortex are programmed to undergo apoptosis in response to ACS, whereas the more mature $CD4^+CD8^-$ or $CD4^-CD8^+$ thymocytes located in the medulla and circulating T lymphocytes are less sensitive to ACS-induced apoptosis.[34-37] Significantly, $P2X_1$ expression was detected only in $CD4^+CD8^+$ thymocytes, but not in peripheral (lymph node) T lymphocytes.[15]

The earlier findings suggesting a role of extracellular calcium in mediating ACS-induced apoptosis of thymocytes are also consistent with evidence that ACS treatment increases the expression of the Type 3 IP_3R in cortical, but not medullary, thymocytes.[16] However, evidence that ACS induce the expression of Type 3 IP_3R on the plasma membrane of S49 cells, a T-cell lymphoma line,[16] appears to be less consistent with earlier evidence indicating the extracellular calcium is not required for induction of apoptosis in lymphoma cells by ACS.[29-32] Moreover, it is possible that the localization of the IP_3R to plasma membrane was a consequence of apoptotic bleb formation in ACS-treated lymphoma cells. The apoptotic blebs that form at the cell surface during apoptosis are membranous structures composed of ER membrane.[38] Thus, further work will be required to determine whether IP_3R induced by ACS treatment are located primarily on the plasma membrane, or are located on the ER membrane and then relocated to the cell surface as apoptotic blebs form.

Although there is considerable evidence that extracellular calcium is not required for apoptosis induction by ACS in lymphoma cells, a role for calcium in mediating ACS-induced apoptosis of lymphoma cells has been supported by several findings. First, calmodulin gene expression is increased in T-cell lymphoma cells after treatment with ACS.[27] Second, calmidazolium interferes with apoptosis in ACS-treated lymphoma cells.[27] Third, stable expression of a cDNA encoding the high-affinity calcium-binding protein, calbindin, inhibited ACS-induced apoptosis in lymphoma cells.[39] Where, then, might the calcium come from that mediates ACS-induced apoptosis in these cells? We and others have detected a diminution of the ER calcium pool in ACS-treated lymphoma cells.[33,40] Thus, one theory is that calcium release from the ER, perhaps via IP_3R located on the ER membrane, may be involved in signaling apoptosis in ACS-treated lymphoma cells.

HOW DOES CALCIUM SIGNAL APOPTOSIS?

Although the novel findings of the past year have provided molecular evidence of a role for calcium in ACS-induced apoptosis, the specific role that calcium plays in death induction and the signal transduction pathway initiated by cytosolic calcium elevation and how it leads to apoptosis are unknown. In the case of T-cell receptor (TCR)-mediated apoptosis, calcium in combination with calmodulin activates calcineurin, a cytosolic protein phosphatase that dephosphorylates and thereby activates the transcription factor $NF\text{-}AT_C$, leading to increased transcription of calcium-regulated, immediate-early genes, including Nur77.[41] Calcineurin function, and hence TCR-mediated apoptosis, is inhibited by the potent immunosuppressants cyclosporin A and FK506.[42] However, these agents do not inhibit ACS-induced apoptosis.[42] Furthermore, ACS- and TCR-mediated apoptotic pathways are mutually antagonistic[42] and calcineurin activation protects T cells from ACS-induced apoptosis.[43] Also, Nur77 is not significantly induced by ACS.[41] One possible lead is a recently identified calcium-binding protein, ALG-2, that has been implicated as necessary for ACS-induced

apoptosis, but its precise role in the apoptotic process has not been defined.[44]

Another concept deserving further consideration is that while calcium mediates apoptosis of ACS-treated cells, it might not be necessary for ACS-induced cell death. This concept is based on a recent report by Iseki et al,[45] who found that high concentrations of the intracellular calcium chelator Quin-2/AM inhibited DNA fragmentation in ACS-treated thymocytes, but did not inhibit cell death. In their hands, calmodulin inhibitors blocked DNA fragmentation, but markedly enhanced cytolysis. One interpretation of these findings is that calcium might be required for DNA cleavage during apoptosis, but not for cell death. Thus, calcium might be involved in endonuclease activation, as part of the degradation phase of apoptosis, but might not be necessary for cell death.

However, the role of calcium in endonuclease activation varies among different types of lymphocytes. Whereas the endonuclease responsible for apoptotic DNA fragmentation in ACS-treated thymocytes requires calcium for maximal activity,[5,13,31,46-49] DNA is cleaved by a calcium-independent endonuclease in the CEM human lymphoblast cell line.[50] These observations correlate with the evidence that extracellular calcium uptake is required for ACS-induced thymocyte apoptosis, but not for ACS-induced apoptosis of lymphoma cell.[12,13,25,28]

Another potential role for calcium in ACS-induced apoptosis might be in protease activation. The calcium-dependent neutral protease, calpain, is activated in the course of apoptosis induction in ACS-treated thymocytes, and a calpain inhibitor appears to inhibit ACS-induced thymocyte cell death.[51] Moreover, destruction of the nuclear structural protein lamin, an event that appears to precede endonucleolytic DNA cleavage during ACS-induced apoptosis, is inhibited by a calpain inhibitor.[52,53] Currently, there is little mechanistic insight into how ACS-induced cytosolic calcium elevation might lead to activation of interleukin-1β converting enzyme–like proteases, which clearly play a prominent role in ACS-induced apoptosis, as well as other forms of apoptosis.[54]

SUMMARY

Since the initial observations by Kaiser and Edelman,[25,28] interest in the role of calcium in ACS-induced apoptosis has wavered, in part because of the fact that extracellular calcium is only necessary for induction of apoptosis in thymocytes, but not in peripheral lymphocytes or lymphoma cells. Now, as a result of molecular evidence implicating two separate ligand-gated calcium channels in ACS-induced apoptosis, interest in the role of calcium is sure to be renewed. The major challenge lies in determining the signal transduction pathway through which ACS-induced calcium fluxes mediate apoptosis.

NOTE ADDED IN PROOF

Recent results (Jayaraman T, Marks AR: *Mol Cell Biol* 17:3005, 1997) indicate that T cells deficient in IP_3RI are resistant to ACS-induced apoptosis, providing additional evidence for a role of calcium in signaling ACS-induced apoptosis.

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