Inhibition of Hepatocytic Autophagy by Adenosine, Aminoimidazole-4-carboxamide Riboside, and N⁶-Mercaptopurine Riboside

EVIDENCE FOR INVOLVEMENT OF AMP-ACTIVATED PROTEIN KINASE

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To examine the role of AMP-activated protein kinase (AMPK; EC 2.7.1.1109) in the regulation of autophagy, rat hepatocytes were incubated with the AMPK proactivators, adenosine, 5-aminooimidazole carboxamide riboside (AICAR), or N⁶-mercaptopurine riboside. Autophagic activity was inhibited by all three nucleosides, AICAR and N⁶-mercaptopurine riboside being more potent (IC₅₀ = 0.3 mM) than adenosine (IC₅₀ = 1 mM). 2'-Deoxycoformycin, an adenosine deaminase (EC 3.5.4.4) inhibitor, increased the potency of adenosine 5-fold, suggesting that the effectiveness of adenosine as an autophagy inhibitor was curtailed by its intracellular deamination. 5-Iodotubercidin, an adenosine kinase (EC 2.7.1.20) inhibitor, abolished the effects of all three nucleosides, indicating that they needed to be phosphorylated to inhibit autophagy. A 5-iodotubercidin-suppressible phosphorylation of AICAR to 5-aminooimidazole-4-carboxamide riboside monophosphate was confirmed by chromatographic analysis. AICAR, up to 0.4 mM, had no significant effect on intracellular ATP concentrations. Because activated AMPK phosphorylates and inactivates 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (EC 1.1.1.88), the rate-limiting enzyme in cholesterol synthesis, the strong inhibition of hepatocytic cholesterol synthesis by all three nucleosides confirmed their ability to activate AMPK under the conditions used. Lovastatin and simvastatin, inhibitors of HMG-CoA reductase, strongly suppressed cholesterol synthesis while having no effect on autophagic activity, suggesting that AMPK inhibits autophagy independently of its effects on HMG-CoA reductase and cholesterol metabolism.

Numerous biochemical processes are involved in the maintenance and support of cell function and cellular growth. These processes need to be coordinated not only relative to each other but also in relation to the metabolic energy available to the cell. The coordination is generally thought to be effectuated in a diffuse fashion by the adenine nucleotides as well as by an upstream kinase (itself activated by AMP) and becomes active when the AMP level is high relative to ATP. Activated AMPK in turn shuts down energy-requiring pathways like fatty acid and cholesterol synthesis through phosphorylation and inactivation of the pertinent key enzymes while indirectly activating an energy-producing pathway like fatty acid oxidation. However, so far the documented regulatory effects of AMPK have been largely confined to lipid metabolism. It would obviously be of interest to study other metabolic pathways to assess the generality of this enzyme’s role in metabolic regulation.

Autophagy occupies a central position in cellular metabolism, supplying small molecules both for anabolic and catabolic purposes through the bulk degradation of proteins and other cellular macromolecules. By a poorly understood sequestration process, pieces of cytoplasm become encapsulated by cellular membranes, forming autophagic vacuoles that eventually fuse with lysosomes to have their contents degraded. The autophagic-lysosomal pathway is subject to complex regulation at the initial sequestration step, by hormones, growth factors, metabolites, and various signaling molecules.

Two previous sets of observations are particularly pertinent to the energy-metabolic control of autophagy; (i) ATP depletion has been shown to reduce the autophagic activity, (4), the overall autophagic-lysosomal protein degradation (5), and the fractional volume of autophagosomes (6) in isolated hepatocytes, apparently by inhibiting several steps in the autophagic-lysosomal pathway (7). (ii) Adenosine and, more potently, N-substituted adenosine analogues are capable of suppressing hepatocytic autophagy (8) and protein degradation (9). It is noteworthy that a large increase in hepatocytic AMP levels is a characteristic feature of both ATP depletion (10–12) and adenosine addition (13). The possibility should, therefore, be considered that AMP, perhaps through activation of AMPK, might be a mediator of autophagy suppression under both conditions. Because autophagy apparently requires energy (4, 7), it would...
seem logical that it be included among the processes shut down by AMPK under conditions of energy depletion (2).

In the present work we have therefore investigated whether the autophagy-inhibitory effect of adenosine might be mediated by its phosphorylation to AMP. Furthermore, we tested the effect of 5-aminocarbazole riboside (AICAR), shown to be a potent activator of AMPK after its intracellular phosphorylation to AICA-ribose or ZMP (19, 15). Both adenosine and AICAR were found to inhibit autophagy strongly. An inhibitor of adenosine kinase (EC 2.7.1.20), 5-iodotubercidin (ITu) (16), suppressed the autophagy-inhibitory effects of both treatments, indicating that formation of AMP and ZMP, respectively, was necessary to inhibit autophagic activity. The results thus support the contention that AMPK may be involved in the regulation of hepatic autophagy.

MATERIALS AND METHODS

**Biochemicals.** [2-14C]Acetic acid (50–62 mCi/mmol, 200 µCi/ml) and [32P]Phosphoric acid (~200 mCi/mmol) were purchased from Amersham International Inc. (Little Chalfont, Buckinghamshire, UK). TLC plates (silica Gel F 1500) were purchased from Schleicher & Schuell (Dassel, Germany). Analytical reagents for lactate dehydrogenase (LDH) (EC 1.1.1.27) measurements were from Boehringer Mannheim (Mannheim, Germany). ATP-monitoring reagents were from Research Biochemicals Inc. (Natick, MA). 2-Deoxycoformycin (DCF) was generously donated by Parke-Davis (Ann Arbor, MI). The luciferin/luciferase assay kit was purchased from LKB-Wallac Oy (Oulu, Finland). Metrizamide was from Nycodens A/S (Ole, Norway). All disposable apparatus for reverse phase HPLC, including 0.45- and 0.22-µm HA filters, were from Millipore (Milford, MA). Collagenase (type IV, from Clostridium histolyticum) and other biochemicals, including AICAR, 5-aminocarbazole riboside (ZMP), N6-mercaptopurine riboside (N6-MPR), and adenosine, were purchased from Sigma Chemical Co. (St. Louis, MO) or from other suppliers of analytical grade chemicals.

**Isolation and Incubation of Hepatocytes.** Hepatocytes were isolated by two-step collagenase perfusion (17) from male albino Wistar rats (250–300 g) fasted for 18 h. After isolation and washing with wash buffer (148 mM Na+, 6.7 mM K+, 1.2 mM Ca++, 151 mM Cl−, pH 7.4) (17), the hepatocytes were suspended in 2-µl aliquots (70–80 mg cellular wet mass/ml) at 37 °C (final concentration, 2% w/v), followed by a 10-min incubation on ice and a 15-min centrifugation at 3700 × g. A fixed volume of supernatant was transferred to a new tube, neutralized with freshly made 1 N KOH, and stored at −20 °C until the measurement day. All samples and solutions used in the HPLC system were filtered through a 0.22-µm Millipore filter.

The mobile phase consisted of two buffers: buffer A (100 mM KH2PO4, pH 5.0, 1.0% acetonitrile, 0.08% tetrabutyl ammonium bromide) and buffer B (150 mM KH2PO4, pH 5.0, 10% acetonitrile, 0.08% tetrabutyl ammonium bromide). The measurement time for each sample was 45 min at a constant flow rate of 1 ml/min with detection at 254 nm. A WISP 710B automated injector and pump, and a Waters multisolenoid delivery system. Data were analyzed by a Waters Maxima 820 chromatography work station.

Inhibition of Autophagy by AMPK-activating Nucleosides—Inhibition of Autophagy by Adenosine: Potentiation by 2′-Deoxycoformycin and Suppression by 5-Iodotubercidin—Previous studies have shown that adenosine and (more potently) some of its N6-substituted derivatives, e.g. N6-dimethyladenosine and N6-methylmercaptopurine riboside, inhibit protein degradation in isolated rat hepatocytes (9). As shown in Fig. 1A, adenosine was able to suppress hepatic autophagy (measured as the sequestration of endogenous LDH), which would explain its degradation-inhibitory ability. The effect of

**Measurement of Intracellular Nucleosides and Nucleotides.**—Intracellular nucleosides and nucleotides were separated and quantified by reverse phase HPLC as described by Shervuch et al. (19) using a reverse phase Supelcosil LC-18-T column (25 cm × 4.6 mm) from SUPELCO (Park Bellefonte, PA). The HPLC instrument system was from Waters (chromatographic division of Millipore Co., Milford, MA), consisting of a Waters 486 tunable absorbance detector adjusted to 254 nm, a WISP 710B automated injector and pump, and a Waters multisolenoid delivery system. Data were analyzed by a Waters Maxima 820 chromatography work station.

After incubation, the samples were immediately treated with 2% perchloric acid and incubated for 15 min at 0 °C. The samples were then centrifuged for 15 min at 3700 × g, the supernatants were transferred to a new set of tubes, neutralized with freshly made 1 N NaOH and stored at −20 °C until the measurement day. Each peak was identified by coelution of the samples with added standards.

**Cholesterol Synthesis.**—Cholesterol synthesis in isolated hepatocytes was measured as the incorporation of [2-14C]acetate according to Rustan et al. (20). After incubation in the presence of [2-14C]acetate (100 µCi, 1 µCi/ml), the cells were washed in 10% isotonic sucrose (4 min at 1600 × g) to remove dead cells and excess [2-14C]acetate. The cells were then dissolved in 1 ml of 50 mM potassium phosphate (pH 7.5) and frozen at −70 °C until the next day, when extraction was performed.

The cell samples were thawed and homogenized by sonication (60 ms/s), and 400 µl of homogenate was transferred to 200 µl of hexane. Cellular lipids were extracted by mixing with 20 times the volume of chloroform/methanol (2.1, v/v), incubation for 30 min, addition of four times the volume of 0.9% NaCl solution, and incubation for 15 min, which allowed the mixture to separate into two phases. After centrifugation (5000 × g for 5 min), the inorganic phase, and the protein layer was gently removed, and the organic phase was dried under nitrogen at 40 °C. The residual lipid extract was redissolved in 200 µl of hexane and separated by thin layer chromatography (acid-resistant silica gel TLC-fol, F 1500, from Schleicher & Schuell) using hexane/diethyl ether/ acetic acid (80:20:1, v/v/v) as the developing system. Lipids were recognized by visualizing added standards in iodine vapor and cut out, and their radioactivity was quantified by liquid scintillation counting in an LKB Wallac beta counter (1281 multigamma).

**RESULTS**

**Inhibition of Autophagy by Adenosine: Potentiation by 2′-Deoxycoformycin and Suppression by 5-Iodotubercidin.—**
Adenosine was strongly potentiated by dCF, a specific adenosine deaminase inhibitor (EC 3.5.4.4) inhibitor (21) that lowered the IC₅₀ of adenosine from 1 to 0.2 mM. The inhibition of autophagy by adenosine would thus normally seem to be restrained by rapid adenosine deamination.

Incubation in the presence of 10 μM ITu, a potent and specific inhibitor of adenosine kinase (16), virtually completely abolished the inhibitory effect of adenosine + dCF on autophagy (Fig. 1B). This absolute requirement for adenosine phosphorylation would suggest that its inhibitory effect on autophagy is mediated by AMP. Measurements of intracellular AMP levels (by HPLC) indeed revealed a 10-fold increase (transiently peaking at 5 μmol/g wet mass) after the addition of adenosine + dCF.²

Inhibition of Autophagy by AICAR and N⁶-Mercaptapurine Riboside—One possible mechanism for the inhibition of autophagy by AMP might be through activation of the AMPK. To investigate this possibility, we incubated hepatocytes with the nucleoside analogue AICAR, which in its monophosphorylated form (AICA-ribotide, or ZMP) is a potent activator of hepatic AMPK (22). As shown in Fig. 2A, AICAR inhibited hepatic autophagy in a dose-dependent manner (IC₅₀ of about 0.3 mM). Its effect on autophagy was completely suppressed by 10 μM ITu, consistent with a requirement for phosphorylation to ZMP. N⁶-MPR, an adenosine analogue thiolated at the N⁶-position, also inhibited autophagic sequestration in an ITu-sensitive manner suggestive of a phosphorylation requirement (Fig. 2B). N⁶-MPR was more potent than adenosine, perhaps because the thiol group at the N⁶-position prevented its deamination by adenosine deaminase, as previously suggested to explain the potent protein degradation-inhibitory effects of other N⁶-substituted adenosine derivatives (9).

The intracellular phosphorylation of AICAR to form ZMP and suppression of this process by ITu were confirmed by reverse phase HPLC analysis of intracellular metabolites in hepatocytes. As shown in Fig. 3A, addition of 0.4 mM AICAR to the hepatocyte suspension resulted in its rapid intracellular accumulation. Accumulated AICAR was effectively phosphorylated to form ZMP, which at 2 h had almost completely replaced

² A. L. Kovács, P. B. Gordon, E. M. Grotterød, and P. O. Seglen, unpublished results.

Fig. 1. Inhibition of autophagy by adenosine: potentiation by deoxycoformycin and prevention by 5-iodotubercidin. A, freshly isolated rat hepatocytes were incubated for 2 h at 37 °C as shaking suspensions at the concentration of adenosine indicated, without (○) or with (●) 50 μM dCF, an adenosine deaminase inhibitor. Each point represents the mean ± S.E./range of two to six independent experiments. B, hepatocytes incubated with the concentration of adenosine indicated plus 50 μM dCF, without (○) or with (●) 10 μM ITu, an adenosine kinase inhibitor. Each value is the mean of triplicate samples from a single experiment. At the end of incubation, the hepatocytes were washed and electrodissrupted. The net amount of LDH autophagically sequestered in sedimentable cell corpses was measured spectrophotometrically and expressed as a percentage of the total cellular LDH.

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Fig. 2. Inhibition of autophagy by adenosine analogues. Hepatocytes were incubated for 2 h at 37 °C with various concentrations of AICAR (A) or N⁶-MPR (B) in the absence (○) or presence (●) of 10 μM ITu. At the end of incubation, the hepatocytes were washed and electrodissrupted. The net amount of LDH autophagically sequestered in sedimentable cell corpses was measured spectrophotometrically and expressed as a percentage of the total cellular LDH. Each point represents the mean ± S.E./range of two to six independent experiments. Some of the error bars are concealed by the symbols.

Fig. 3. Inhibition by 5-iodotubercidin of intracellular phosphorylation of AICAR to form ZMP. Hepatocytes were incubated at 37 °C with AICAR alone for 2 min (A) or 2 h (B) or together with 10 μM ITu for 2 h (C). At the end of incubation, the cells were precipitated with perchloric acid (final concentration, 2% w/v) and neutralized with NaOH. Nucleosides and nucleotides in the neutralized perchloric acid extracts were separated by reverse phase HPLC using a Supelcosil LC-18-T column and identified by internal standards (not shown). Chromatograms in A, B, and C are representative of several independent experiments with identical results.

AICAR (Fig. 3B). This phosphorylation was suppressed by the addition of 10 μM ITu (Fig. 3C), indicating the involvement of adenosine kinase.
**FIG. 4.** Effects of AICAR on hepatocellular ATP content. The ATP content of hepatocytes incubated for 2 h at 37 °C with different concentrations of AICAR was measured in neutralized perchloric acid extracts, using a luminometer and a luciferin/luciferase assay. Each point represents the mean ± S.E.range of two to five experiments.

**Effect of AICAR on the Intracellular ATP Concentration**—Autophagic sequestration has previously been shown to correlate positively with intracellular ATP levels (4), suggesting a requirement for energy. To check whether AICAR might inhibit autophagy by altering the intracellular ATP concentration, its effect on hepatocellular ATP levels was measured. As shown in Fig. 4, AICAR concentrations below 0.6 mM had no effect on intracellular ATP levels, although autophagic sequestration was inhibited 80%. At higher AICAR concentrations, some reduction in ATP (about 30% at 1 mM) was observed. Apparently, AICAR does not exert its inhibitory effect on autophagy by altering intracellular ATP.

**Effects of Adenosine, AICAR, and N6-MPR on Hepatocellular Cholesterol Synthesis**—One of the established biological roles of activated AMPK is to phosphorylate and inactivate HMG-CoA reductase (EC 1.1.1.88), the rate-limiting enzyme in cholesterol synthesis (23, 24). If adenosine, AICAR, and N6-MPR really activate AMPK, they would therefore be expected to inhibit hepatocellular cholesterol synthesis. As shown in Fig. 5, all these compounds inhibited cholesterol synthesis in isolated hepatocytes, with about the same potencies as in their inhibitory effect on hepatocellular ATP content. Inhibition of autophagy by AMPK activators of the AMPK cascade involved in the regulation of lipid metabolism (8, 9, 34) have been implicated in the regulation of autophagy.

**DISCUSSION**

Autophagy, or macroautophagy, is a cellular process that plays a major role in the bulk sequestration and subsequent lysosomal degradation of long-lived cytosolic proteins, ribosomal RNA, and cytoplasmic organelles (3, 26–28). The initial autophagic sequestration step is highly regulated by growth factors, hormones, and metabolites such as amino acids and adenosine (which can be regarded as negative feedback inhibitors of autophagy) and by protein phosphorylation (8, 9, 29–31). A type 2A protein phosphatase (32) as well as the Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (31), the AMP-dependent protein kinase (33), and protein tyrosine kinase activity (34) have been implicated in the regulation of autophagy in isolated rat hepatocytes.

Depletion of ATP inhibits autophagic sequestration, suggesting that autophagy is an energy-dependent process (4, 7). However, ATP depletion also leads to increases in intracellular AMP and adenosine (10, 12). Because adenosine, adenosine, and some of their thiolated or methylated derivatives also inhibit autophagic protein degradation (8, 9, 35, 36), the possibility should be considered that at least part of the autophagy-inhibitory effect of ATP depletion might be mediated by adenosine or AMP, perhaps through AMPK.

AMPK is the central component in a multisubstrate protein kinase cascade involved in the regulation of lipid metabolism (37). AMPK is activated in response to elevations of the AMP/ATP ratio, caused e.g. by hypoxia, environmental stress, star-
vation, cell damage, heat, etc. (23, 38). Upon its activation, AMPK shuts down major ATP-consuming biosynthetic processes like cholesterol and fatty acid synthesis, thereby preserving ATP for more essential and immediate cellular needs such as the maintenance of ion gradients. It has been suggested that AMPK may serve as a general integrator of metabolic responses to changes in energy availability likely to have regulatory effects extending beyond lipid metabolism (2).

In the present study, the ability of the adenosine kinase inhibitor ITu (16) to abolish the autophagy-suppressive effect of adenosine clearly shows that the suppression requires AMP formation and most likely is mediated by AMP. This effect of ITu would effectively rule out the possibility that adenosine might mediate its effect through purinergic (adenosine) receptors, known to be present at the surface of rat hepatocytes (39, 40). The administration of adenosine alone has previously been shown to raise the concentration of AMP in isolated rat hepatocytes (13). Under the conditions used in the present experiments, with the adenosine and adenylyl deaminase inhibitor, dCF (21), included to prevent the deamination of adenosine and AMP, a rapid increase in intracellular AMP to very high levels has been demonstrated. The marked synergism between adenosine and dCF in suppressing autophagy would thus be consistent with a mediation by AMP. Both adenosine and dCF are also capable of elevating the hepatocytic levels of other adenine metabolites, like S-adenosylhomocysteine and (secondarily) S-adenosylmethionine (41, 42), but this pathway would be potentiated rather than antagonized by ITu (43).

AMP can directly activate or inactivate a number of enzymes, e.g. in glucose/glycogen metabolism (2, 13); the ability of AMP to inhibit autophagy does not, therefore, prove that AMPK is involved. However, the equally effective inhibition of autophagy by AICAR would more strongly implicate AMPK. AICAR becomes a potent and specific activator of AMPK after its intracellular phosphorylation to AICA-ribotide or ZMP (14, 15, 22, 44). AICAR was rapidly converted to ZMP under the experimental conditions. AICAR, at concentrations that inhibit hepatic cholesterol synthesis, as an indirect measure of in situ AMPK activity, N⁶-MPR 5'-monophosphate did not activate purified rat liver AMPK, in contrast to AMP and ZMP. To see if the situation might be different in intact cells, we measured the effect of N⁶-MPR on hepatic cellular cholesterol synthesis, as an indirect measure of in situ AMPK activity. N⁶-MPR was found to strongly inhibit cholesterol synthesis, and in an ITu-sensitive manner, suggesting that its 5'-monophosphate was in fact able to activate AMPK in intact cells. The discrepancy between this observation and the inability of N⁶-MPR 5'-monophosphate to activate purified AMPK can perhaps be explained by the complex manner in which AMPK is regulated. The direct allosteric activation of AMPK by 5'-nucleotides may increase its activity maximally 5-fold (46), whereas its phosphorylation by an upstream AMP-activated protein kinase kinase may cause a more than 20-fold activation (47, 48). These two effects can act synergistically to produce, potentially, a more than 50-fold activation of AMPK (48). Because the effects of 5'-nucleotides on the two enzymes are independent (49), it is theoretically quite possible for a given nucleotide, like N⁶-MPR, to cause a substantial activation of AMPK by binding to the upstream kinase rather than to AMPK itself.

In the present work, hepatocellular cholesterol synthesis has been used as an indirect measure of AMPK activity. However, the data also demonstrate that all the investigated nucleosides cause a strong suppression of HMG-CoA reductase activity and of cholesterol formation, raising the possibility that the latter effects could be instrumental in mediating the inhibition of autophagy. The effects of lovastatin and simvastatin, two potent and specific inhibitors of HMG-CoA reductase (25), were therefore investigated. Both inhibitors completely blocked cholesterol synthesis while having no effect on autophagy. It would thus seem clear that neither HMG-CoA reductase activity nor de novo cholesterol formation is required for autophagic activity. The effects of adenosine, AICAR, and N⁶-MPR on cholesterol metabolism and on autophagy should probably be regarded as two independent consequences of AMPK activation: HMG-CoA reductase phosphorylation/inhibition and modification of another as yet unknown protein.

In conclusion, the present results suggest that hepatocytic autophagy can be suppressed by AMP through activation of AMPK. The adenosine-antagonistic effects of ITu would rule out a direct regulation by adenosine, making it unlikely that adenosine (an RNA degradation product) can function as a physiological feedback inhibitor of autophagic degradation. Although 0.5 mM added adenosine could elevate intracellular AMP levels dramatically in the presence of dCF, physiological adenosine concentrations would probably be too low to have much regulatory effect in the absence of inhibitors. On the other hand, high levels of AMP can be reached under hypoxia and other conditions of energy depletion (10–13), which also cause a suppression of autophagy (4–7). The physiological relevance of the regulation of autophagy by AMP/AMPK may therefore be to shut down this energy-requiring process under conditions of energy depletion.

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