The Adipokine Lipocalin 2 Is Regulated by Obesity and Promotes Insulin Resistance

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OBJECTIVE—We identified lipocalin 2 (Lcn2) as a gene induced by dexamethasone and tumor necrosis factor-α in cultured adipocytes. The purpose of this study was to determine how expression of Lcn2 is regulated in fat cells and to ascertain whether Lcn2 could be involved in metabolic dysregulation associated with obesity.

RESEARCH DESIGN AND METHODS—We examined Lcn2 expression in murine tissues and in 3T3-L1 adipocytes in the presence and absence of various stimuli. We used quantitative Western blotting to observe Lcn2 serum levels in lean and obese mouse models. To assess effects on insulin action, we used retroviral delivery of short hairpin RNA to reduce Lcn2 levels in 3T3-L1 adipocytes.

RESULTS—Lcn2 is highly expressed by fat cells in vivo and in vitro. Expression of Lcn2 is elevated by agents that promote insulin resistance and is reduced by thiazolidinediones. The expression of Lcn2 is induced during 3T3-L1 adipogenesis in a CCAAT/enhancer-binding protein–dependent manner. Lcn2 serum levels are elevated in multiple rodent models of obesity, and forced reduction of Lcn2 in 3T3-L1 adipocytes improves insulin action. Exogenous Lcn2 promotes insulin resistance in cultured hepatocytes.

CONCLUSIONS—Lcn2 is an adipokine with potential importance in insulin resistance associated with obesity. Diabetes 56:2533–2540, 2007

The worldwide epidemic of obesity and type 2 diabetes has focused attention on adipocyte biology and the role of adipose tissue in the integration of systemic metabolism (1). The discovery of leptin more than a decade ago established a paradigm in which secreted proteins from adipocytes coordinate energy balance and glucose homeostasis (2,3).

Since that initial discovery, the number of adipocyte-derived signaling molecules has grown ever larger, and the term adipokine was coined to reflect that many of these molecules exert positive or negative actions on inflammation. Several adipokines promote insulin sensitivity, including leptin (2), adiponectin (4), and visfatin (5), while others induce insulin resistance, such as resistin (6) and retinol binding protein (RBP)4 (7).

Lipocalin 2 (Lcn2)—also known as neutrophil gelatinase–associated lipocalin, siderocalin, and 24p3—is a member of a large superfamily of proteins that includes RBP4. Lipocalins are small generally secreted proteins with a hydrophobic ligand binding pocket (8). Known ligands for lipocalins include retinol, steroids, odorants, pheromones, and, in the case of Lcn2, siderophores (9).

Siderophores are small molecules used by bacteria to poach iron from their hosts, a necessary cofactor for the growth of some pathogens. Lcn2 is used by the mammalian innate immune system to sequester siderophore and thus deprive the bacteria of iron. Mice lacking Lcn2 appear normal but die when exposed to siderophore-requiring strains of bacteria in quantities that are cleared easily by wild-type mice (10,11). Lcn2 can thus be considered an iron transport protein, and it has been implicated in the apoptotic induction of pro-B-cells (12) and in the biology of the genitourinary system, both as a developmental factor and as a protective mechanism in renal ischemia (13).

In this study, we identify Lcn2 as a factor dramatically induced by dexamethasone (Dex) and tumor necrosis factor (TNF)-α in 3T3-L1 adipocytes and show that adipose tissue is a dominant site of Lcn2 expression in the mouse. We also study the regulation of Lcn2 expression in adipocytes and demonstrate that it is regulated by obesity. We also provide data suggesting that Lcn2 promotes insulin resistance in adipocytes.

RESEARCH DESIGN AND METHODS

Cell culture and differentiation. 3T3-L1 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% BCS at 5% CO2. Once confluence was reached, cells were exposed to DMEM with 10% fetal bovine serum (FBS) containing a differentiative cocktail including Dex (1 μmol/l), insulin (5 μg/ml), and isobutylmethylxanthine (0.5 mmol/l). After 2 days, cells were maintained in medium containing insulin until ready for harvest at day 7. NIH-3T3 cells were maintained in the same conditions. In some experiments, 3T3-L1 cells were differentiated only with 5 μmol/l rosiglitazone plus 10% FBS, which was changed every 2 days. H11E rat hepatoma cells were cultured in αMEM medium (Invitrogen), supplemented with 10% FBS at 37°C with 5% CO2. Cells were seeded in 24-well plates with 50% confluence. Before treatment, cells were washed twice with αMEM containing 0.2% FBS and cultured overnight (18 h) in medium supplemented with or without recombinant Lcn2 (10 nmol/l)—with or without Lcn2 (10 nmol/l)—with Dex treatment (250 nmol/l) in parallel as a positive control. The next day, cells were treated with 100 nmol/l insulin for 30 min, then washed twice with Krebs-Ringer HEPES buffer plus lactate and pyruvate (10 mmol/l HEPES, pH 7.4, 96 mmol/l NaCl, 1.25 mmol/l CaCl2, 1.25 mmol/l MgSO4, 1.2 mmol/l KHPO4, 25 mmol/l NaHCO3, 20 mmol/l lactate, and 2 mmol/l pyruvate). Cells were incubated in Krebs-Ringer HEPES buffer and supplemented with Lcn2, Dex, or carrier solution for another 6 h at 37°C. Supernatants were collected for the glucose oxidase assay, and cells were harvested by Trizol (Invitrogen) for RNA analysis.
Recombinant Lcn2 was produced as described (13). Endotoxin was assayed in 100 μl of 1 mmol/l recombinant Lcn2 solution by means of a limulus amoebocyte lysate gel clot assay (0.125 EU/ml sensitivity; Cambrex/Lonza, Allendale, NJ) and was found to be below the limits of detection for the assay.

**Animals.** All animal studies were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. For the high-fat diet studies, 3- to 4-week-old FVB male mice were obtained from Taconic. Mice were fed a standard Chow diet (Formulab 5008) or high-fat diet (55% fat calories; Harlan-Teklad 93075). Animals were put on diet treatment at 4–5 weeks of age. To induce obesity, mice were treated with ob/ob (C57BL/6J mice). For each sample, 10 μg total RNA was loaded onto formaldehyde-agarose gels, transferred onto nylon membranes, and hybridized with the appropriate 32P-labeled probe in Ultrahyb (Ambion).

**Quantitative PCR.** First-strand cDNA synthesis for quantitative PCR was performed using RETROscript (Ambion). Total RNA (1.5 mg) was converted into first-strand cDNA using oligo dT primers as described in the kit. cDNA was amplified and detected with the Brilliant SYBR Green PCR master mix (Stratagene). Real-time PCR was performed in an Mx3000P thermocycler (Stratagene), and its software was used to calculate the cycle threshold of each reaction. Validation experiments were performed to demonstrate equal efficiencies of target Lcn2 and internal control (188 RNA for cDNA synthesis and of 373.4 for C57BL/6 mice). The relative amount of Lcn2 transcripts was determined using comparative C method with the expression level of untreated control as 1. Primer sequences are as follows:

- mG6Pase-F, GACACGCTCTCGTCTTCCAGCA; mG6Pase-R, GATAGGGAGTCTGAAC
- mFabp4-F, GCATGGTGAGGCTTGTGGAA; mFabp4-R, CTTGTGGAAGTCACGCCTTT
- mPparg-F, GCATGGTGAGGCTTGTGGAA; mPparg-R, CTTGTGGAAGTCACGCCTTT
- mLeptin-F, CAGTGA; mLeptin-R, TCATTGGCTATCTGCAGCAC

**Northern blotting.** Cells were lysed in Trizol and processed according to the manufacturer's instructions. Murine tissues were harvested from wild-type females and lean littermate controls (n = 8 each). Total RNA (1.5 mg) was converted into first-strand cDNA using oligo dT primers as described in the kit. cDNA was amplified and detected with the Brilliant SYBR Green PCR master mix (Stratagene). Real-time PCR was performed in an Mx3000P thermocycler (Stratagene), and its software was used to calculate the cycle threshold of each reaction. Validation experiments were performed to demonstrate equal efficiencies of target Lcn2 and internal control (188 RNA for cDNA synthesis and of 373.4 for C57BL/6 mice). The relative amount of Lcn2 transcripts was determined using comparative C method with the expression level of untreated control as 1. Primer sequences are as follows:

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- mPparg-F, GCATGGTGAGGCTTGTGGAA; mPparg-R, CTTGTGGAAGTCACGCCTTT
- mLeptin-F, CAGTGA; mLeptin-R, TCATTGGCTATCTGCAGCAC

**Glucose oxidase assay.** For the glucose oxidase colorimetric method, we used the Amplicor Red glucose/glucose oxidase assay kit, following the manufacturer's instructions. Absorption at 571 nm was measured in a PowerWave XS microplate spectrophotometer (BioTek). This experiment was performed in triplicate (three wells for each condition).

**RESULTS**

Lcn2 expression in 3T3-L1 adipocytes is induced by Dex and TNF-α. We performed a genomic screen to identify common mechanisms of insulin resistance, using Dex and TNF treatment of 3T3-L1 adipocytes as a model system. The major outcome of this study was the observation that genes associated with reactive oxygen species were affected concordantly by these two highly disparate treatments (14). Lcn2 was another gene induced strongly by both TNF and Dex in the microarray experiment. This effect was confirmed by quantitative PCR, which showed induction of Lcn2 mRNA of ~80-fold by Dex and 30-fold by TNF (Fig. 1A). The effect of TNF was also seen

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previously (16). The insulin-sensitizing agent rosiglitazone significantly attenuated Lcn2 mRNA expression by either agent.

**Lcn2 is highly expressed in adipocytes in vitro and in vivo.** Others have reported Lcn2 expression in fat (16–19), but there has been no attempt to compare adipose expression to other sites. Northern analysis showed that white adipose tissue (WAT) was by far the dominant site of expression of Lcn2 in wild-type male mice (Fig. 1B). We also saw significant amounts of Lcn2 mRNA in lung and in testis/epididymis—both reported as major sites of expression (20). We next sought to determine whether Lcn2 expression is regulated during adipogenesis. 3T3-L1 preadipocytes were differentiated using a standard cocktail containing Dex, methylisobutylxanthine, and insulin, and Lcn2 expression was assessed with quantitative PCR at various time points. We noted an immediate and profound induction of Lcn2 mRNA within the first day of differentiation (Fig. 2A); levels remained elevated for at least 7 days. The Ct for Lcn2 in mature 3T3-L1 adipocytes ranges from 21 to 23; the corresponding value in murine WAT is 18. We looked at the contribution of each component of the induction cocktail (Fig. 2B) and found that Dex was the dominant contributor to Lcn2 induction, as expected. However, methylisobutylxanthine also had a significant effect on Lcn2 levels, and the combination of Dex and methylisobutylxanthine was maximally potent, with no significant contribution from insulin. We were interested to know whether Lcn2 expression in 3T3-L1 cells was dependent on the specific induction cocktail or whether it was linked to adipogenesis per se. This was addressed by differentiating 3T3-L1 cells in the absence of Dex/methylisobutylxanthine, or insulin, using rosiglitazone only. Lcn2 expression rose more gradually than when Dex/methylisobutylxanthine were present (Fig. 2C) but reached similar levels later during differentiation. The apparent contradiction between the effect of rosiglitazone in Figs. 1 and 2 is resolved by considering the developmental status of the cells; in undifferentiated cells, rosiglitazone promotes adipogenesis and thus indirectly promotes Lcn2 expression. In mature cells, however, the direct effect of rosiglitazone is suppression of Lcn2 expression.

**Expression of Lcn2 in adipocytes is C/EBP dependent.** Many adipocyte genes are transcriptionally regulated by peroxisome proliferator–activated receptor
(PPAR)γ and/or members of the C/EBP family of bZIP proteins (18). The ability of rosiglitazone to repress Lcn2 (Fig. 1A) suggested that PPARγ was unlikely to be a direct inducer of Lcn2 expression. We thus tested whether C/EBP isoforms might serve this purpose. C/EBPα, -β, and -δ, delivered by retroviral transduction, were all effective at inducing endogenous Lcn2 expression in PPARγ−/− fibroblasts (Fig. 3A). These cells were chosen to avoid the confounding effects of simultaneous adipogenesis; C/EBPs cannot induce differentiation in the absence of PPARγ (14). To identify C/EBP binding sites in the Lcn2 promoter, we performed a computational search (Fig. 3B). This revealed a possible C/EBP site with a high degree of conservation between mouse, rat, and humans at −218 of the murine promoter. Trans-activation assays in NIH-3T3 cells showed that the ability of C/EBPδ to induce expression of this construct dropped off sharply when deletions were made that eliminated this site (Fig. 3C). The same effect was seen with C/EBPα and -β (data not shown). Mutation of the core sequence of this site from TTGC to GGGA significantly decreased the ability of C/EBPδ to trans-activate the reporter (Fig. 3D). Finally, we used Chromatin Immunoprecipitation to demonstrate specific C/EBP isoform binding to this element in living cells (Fig. 3E). Before differentiation, no C/EBP isoform was bound to the site, but C/EBPβ and -δ were highly bound by the first day after induction. By day 4, C/EBPα binds the site as well, which is consistent with the delayed appearance of this factor during 3T3-L1 adipogenesis (21), followed by a reduction in C/EBPβ and -δ binding, which reflects their diminished expression.
Lcn2 levels are elevated in obesity. We next looked at whether Lcn2 expression is altered by obesity. Western blotting of lysates from the adipose tissue of obese (ob/ob) mice revealed a significant elevation of Lcn2 relative to lean controls (Fig. 4A). We also examined adipose tissue from mice fed either a chow or high-fat diet after fractionation into mature adipocytes, SVCs, and macrophages (Fig. 4B). Lean animals (30.3 ± 0.4 g; n = 9) had equivalent Lcn2 mRNA expression in the adipocyte and SVC fractions, while obese animals (53.0 ± 0.10 g; n = 8) shifted Lcn2 mRNA expression away from the SVC fraction and toward mature adipocytes. There was no significant expression of Lcn2 in adipose tissue–resident macrophages in either the lean or obese state. We were somewhat surprised to find significant Lcn2 expression in the stromal vascular fraction (SVF) of lean animals, given the low levels seen in cultured preadipocytes. There are two plausible explanations for this. First, the low-speed centrifugation method used to separate adipocytes from SVF may not separate cells that are early in the differentiation process (i.e., before significant lipid accumulation). Since Lcn2 appears to be induced early in differentiation, this could account for a higher-than-expected amount of Lcn2 in the SVF. Alternatively, there may be significant Lcn2 expression in other cell types in the SVF (e.g., endothelial cells or fibroblasts). Consonant with the data from ob/ob mice, Lcn2 protein expression was elevated in WAT of high-fat–fed animals (data not shown). Given the elevated expression in adipose tissue, we next assessed whether increased serum levels of Lcn2 are associated with excess adiposity. In fact, we found elevated serum levels of Lcn2 in three different murine obesity models. Lcn2 was increased relative to lean controls in ob/ob mice (1.5 ± 0.40-fold, P = 0.02) (Fig. 4C), db/db mice (3.3 ± 1.8-fold, P = 0.01) (Fig. 4D), and in high-fat feeding (3.6 ± 5.6-fold, P = 0.03) (Fig. 4E). All of these samples were collected in the fed state to reduce confounding due to possible nutritional influences on Lcn2 levels. Nonetheless, Lcn2 levels are still elevated in obese db/db mice even in the fasted state (13.6 ± 4.2-fold, P = 0.01). The body weights of these mice were as follows: 31.6 ± 0.8 g (chow) vs. 39.9 ± 0.7 g (high-fat diet).

FIG. 4. Lcn2 is elevated in obesity. A: Lcn2 protein levels in perigonadal WAT lysates (30 μg/lane) from male ob/+ (n = 5) and ob/ob (n = 7) mice. Data are means ± SD. *P < 0.05. B: Lcn2 mRNA expression in fractionated WAT from male C57BL mice given chow (n = 7) or high-fat (n = 7) diet, relative to expression in chow macrophages. Ads, adipocytes; Macs, macrophages. Data are means ± SD. *P < 0.05, ***P < 0.001. C: Lcn2 protein levels in serum from fed male ob/+ (n = 6) and ob/ob (n = 10) mice, measured by Western blotting and expressed as fold relative to the mean of ob/+ controls. D: Lcn2 protein expression in serum from chow (n = 15) and high-fat–fed male (n = 18) mice, expressed as fold relative to the mean of chow-fed controls. Data for C, D, and E are shown as the mean for each group, with representative Western blots from three lean and three obese animals shown on top. For SD and statistical analysis, see text.
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21.0 ± 0.45 g (db/+) vs. 41.3 ± 0.85 g (db/db), and 26.0 ± 0.51 g (ob/+) vs. 47.7 ± 3.51 g (ob/ob).

Lcn2 promotes insulin resistance in cultured adipocytes and hepatocytes. Several factors converge to suggest that Lcn2 may promote insulin resistance, including serum elevation in obesity, induction by TNF and Dex, repression by thiazolidinediones, and structural similarity to RBP4. We attempted to test this directly by adding purified Lcn2 to mature 3T3-L1 adipocytes and then measuring insulin-stimulated glucose uptake, but we were unable to find a consistent change in glucose uptake in the presence of Lcn2, either as apo-Lcn2 or after the protein was incubated with a siderophore-iron complex (data not shown). We were concerned that this might reflect that Lcn2 is not limiting in the culture medium of 3T3-L1 adipocytes, which produce and secrete large amounts of the protein. The amount of Lcn2 in conditioned medium is similar to that seen in the serum of obese mice (data not shown). We thus approached this issue from a different direction by asking whether reducing Lcn2 levels lead to improved insulin action. This was accomplished through retroviral delivery of short hairpin (sh)RNA directed against Lcn2. We identified a hairpin that reduced expression of Lcn2 by >90%, as measured by quantitative PCR (Fig. 5A) or Western blot (Fig. 5B). Importantly, cells expressing this shRNA were differentiated to the same degree as cells expressing a control hairpin, as determined by oil red O staining of lipid accumulation (Fig. 5C) and marker expression (Fig. 5A and B). Cells expressing the Lcn2 shRNA, however, showed elevated glucose uptake in both the basal and insulin-stimulated states (Fig. 5D). Importantly, the component of glucose uptake that reflects insulin action (i.e., the difference between the insulin-stimulated and the basal glucose uptake) was significantly elevated in cells expressing the Lcn2 shRNA (Fig. 5E).

We next tested whether exogenous Lcn2 could affect insulin sensitivity in cultured H4IIe hepatocytes. In the absence of insulin, Lcn2 did not affect glucose production (Fig. 6A) or glucose 6-phosphatase expression (Fig. 6B and C). Lcn2 did, however, partially block the suppressive effects of insulin on these parameters. No effect of Lcn2 was seen on PEPCK mRNA levels, either in the presence or absence of insulin (data not shown). Importantly, the magnitude of insulin resistance induced by Lcn2 in these cells was comparable to that achieved with Dex. Interestingly, apo-Lcn2 (i.e., not complexed with siderophore and iron) was unable to induce insulin resistance in cultured hepatocytes (Fig. 6D).

DISCUSSION

It is now appreciated that adipocytes secrete a wide array of proteins that influence systemic metabolism. These include factors that promote insulin sensitivity as well as others that induce insulin resistance (3). We show here that Lcn2 is highly expressed in adipocytes, that its expression is regulated by obesity, and that it induces insulin resistance. In this sense, it behaves in a very similar fashion to RBP4, another member of the lipocalin superfamily and a close relative of Lcn2. While others have
noted adipose expression of Lcn2 (16–19), our data are the first to demonstrate that adipocytes may be the dominant source of Lcn2 expression. Furthermore, we show that adipose-specific expression is dictated in large part by C/EBP-dependent trans-activation of a defined element in the Lcn2 promoter. The lack of Lcn2 in brown adipose tissue is interesting and implies that WAT-specific factors besides C/EBP are required or that brown adipose tissue contains specific repressors of Lcn2 synthesis.

Lcn2 has been proposed to serve many functions, ranging from apoptosis to uterine involution to genitourinary development (12,22,23). Data obtained from knockout mice, however, suggest that Lcn2 serves as part of the innate immune system used as a nonspecific defense against microbes (10,11). In this capacity, Lcn2 expression occurs in inflamed epithelial tissues in direct contact with potential pathogens, such as respiratory and intestinal epithelium (24). Adipose tissue is not usually considered to be in direct contact with invading pathogens, but a large body of data has now accumulated suggesting that fat is intimately involved in immune activity and acute-phase response. Furthermore, obesity is considered to be a pro-inflammatory state with elevation of multiple markers of inflammation (25); increased Lcn2 seen in obese animals is consonant with this idea.

Based on the studies presented here, we propose that Lcn2 acts as an adipocyte-derived mediator of insulin resistance. This assertion is found on several lines of evidence, both direct and indirect. First, agents that promote insulin resistance induce the expression of Lcn2, including glucocorticoids and TNF-α. Similarly, hyperglycemia, which also reduces insulin sensitivity in adipocytes, causes enhanced expression of Lcn2 in adipocytes (26). Second, insulin-sensitizing thiazolidinedione compounds reduce the expression of Lcn2 in adipocytes (Fig. 4) (27). Third, Lcn2 is elevated in multiple murine models of obesity. Finally, reduction of Lcn2 in cultured adipocytes improved insulin sensitivity, demonstrating a direct link between this secreted molecule and cellular glucose homeostasis. The fact that exogenous Lcn2 did not affect glucose uptake in 3T3-L1 adipocytes (data not shown) is interesting and suggests that Lcn2 levels in media conditioned by the cultured adipocytes are already so high that adding more has no incremental effect. Interestingly, data from db/db mice (16,27) indicate that Lcn2 expression is elevated in the liver in this obese model; our data suggest that liver Lcn2 expression trends lower in high-fat–fed mice (data not shown). Thus, the contribution of extra-adipose sources of Lcn2 to serum is unclear and may differ between obesity models.

How might Lcn2 act to induce insulin resistance? Lcn2 has been proposed to be an iron delivery protein (22), and there is a well-known association between iron accumulation and diabetes (28). Patients with hemochromatosis, for example, have insulin resistance in addition to reduced insulin secretory capacity (28), and iron intake in healthy women has been positively associated with the risk of developing type 2 diabetes (29). Most of this effect has been inferred to involve hepatic insulin sensitivity (28); iron-mediated dysregulation of insulin action in adipocytes has never been explicitly assessed. Adipocytes certainly express iron-regulatory proteins, however, and iron has

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**FIG. 6.** Exogenous recombinant Lcn2 induces insulin resistance in H4IIe hepatocytes. A, left: Glucose production induced by liganded Lcn2 (10 nmol/l) or Dex (250 nmol/l) in the presence or absence of insulin (100 nmol/l). A, right: Effect of liganded Lcn2 (10 nmol/l) or Dex (250 nmol/l) on glucose-6-phosphatase mRNA expression in the presence or absence of insulin (100 nmol/l). B: Dose response of liganded Lcn2 on glucose-6-phosphatase expression. C: Effect of apo-Lcn2 on glucose-6-phosphatase expression. Data are means ± SD, *P < 0.05, **P < 0.01, n = 3.
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been shown to mediate lipolysis in cultured fat cells (30). Consistent with the idea that iron is required for the effect of Lcn2 on insulin action, apo-Lcn2 was ineffective in causing insulin resistance in cultured hepatocytes. Iron may induce insulin resistance through the formation of specific reactive oxygen species, given the well-studied role of transition metals in catalyzing the Fenton reaction in cells. We have shown that reactive oxygen species act causally in multiple forms of insulin resistance in mice and in cultured adipocytes (15).

Lcn2 may also signal through a specific receptor, which may or may not involve the subsequent delivery of iron. Green and colleagues (31) cloned an organic cation transporter that is suggested to mediate Lcn2 internalization and downstream functions in multiple cell types. We have confirmed the expression of this molecule in brain and liver using Northern analysis and PCR but do not detect it in adipose samples.

Our data suggest that Lcn2 may be added to the growing list of secreted molecules that adipocytes use to modulate glucose homeostasis. We are now testing this hypothesis directly in vivo in mice using both gain-of-function and loss-of-function approaches.

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