Glucagon increases hepatic mRNA concentrations of ureagenic and gluconeogenic enzymes in early-lactation dairy cows

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ABSTRACT

Exogenous glucagon increases hepatic glucose synthesis in part by increasing hepatic extraction of amino acids from blood for conversion to glucose. To examine the role of glucagon in orchestrating gene expression of gluconeogenic and ureagenic enzymes, we determined the mRNA concentrations of key hepatic ureagenic and gluconeogenic enzymes at d 11, 15, and 22 postpartum in multiparous Holstein cows that received 0 or 5 mg of glucagon in 60 mL of saline by subcutaneous injection every 8 h for 14 d starting on d 8 postpartum. On d 11 postpartum, glucagon increased the hepatic mRNA concentrations for all measured ureagenic enzymes (carbamoylphosphate synthetase I, ornithine transcarbamylase, and argininosuccinate synthetase) and gluconeogenic enzymes (pyruvate carboxylase and cytosolic and mitochondrial forms of phosphoenolpyruvate carboxykinase) and increased or tended to increase mRNA concentrations of gluconeogenic enzymes on d 15 postpartum but not on d 22. The effect of glucagon to increase mRNA concentrations of ureagenic and gluconeogenic enzymes was limited to times when concentrations of plasma insulin were not increased. Our results suggest that hepatic gene expression of key ureagenic and gluconeogenic enzymes in early-lactation dairy cows is responsive to hormonal regulation by glucagon.

Key words: gene expression, glucagon, gluconeogenesis, ureagenesis

INTRODUCTION

Multiple s.c. injections of glucagon improve the carbohydrate status of early-lactation dairy cows by increasing hepatic glucose synthesis (Bobe et al., 2003a;

Nafikov et al., 2006; Osman et al., 2008). One biological mechanism by which glucagon increases hepatic glucose synthesis is by increasing hepatic extraction of circulating amino acids for conversion to glucose (Brockman et al., 1975). In vitro, glucagon increases ureagenesis in bovine hepatocytes (Zhu et al., 2000). Key regulatory enzymes of ureagenesis are argininosuccinate synthetase (**ASS**), carbamoylphosphate synthetase I (**CPS-I**), and ornithine transcarbamylase (**OTC**). Their activities are regulated in part by their transcription rates. In rat liver and hepatocytes, their transcription rates are increased by glucagon through its secondary messenger cyclic AMP (Ryall et al., 1986; Morris et al., 1987; Ulbright and Snodgrass, 1993). To our knowledge, the effects of glucagon on mRNA concentrations of ureagenic enzymes in dairy cows have not been reported.

The effect of glucagon on activities and mRNA concentrations of gluconeogenic enzymes such as pyruvate carboxylase (**PC**) and phosphoenolpyruvate carboxykinase (**PEPCK**), which is present in cytosolic (**PEPCK-C**) and mitochondrial forms (**PEPCK-M**), in ruminants have been inconclusive. In mid-lactation cows, Williams et al. (2006) reported a 1.5-fold increase in mRNA concentrations of PC, PEPCK-C, and PEPCK-M in response to subcutaneously injected glucagon. However, She et al. (1999) reported that intravenously infused glucagon increased mRNA concentrations of PC but decreased mRNA concentrations of PEPCK-C. One reason for the inconclusive results might be insulin, which is released from the pancreas in response to elevated glucagon concentrations (Burcelin et al., 1996). Glucagon increases in vitro gluconeogenesis in bovine hepatocytes; however, this response is counteracted by insulin (Donkin et al., 1997). In rat liver and hepatocytes, glucagon increases, through its secondary messenger cyclic AMP, mRNA concentrations of PEPCK-C, which are attenuated or even counteracted by insulin in a dose-dependent manner (Beale et al., 1984; Christ et al., 1988, 1990).

The objective was to evaluate the role of glucagon administered as multiple s.c. injections in orchestrating gene expression of gluconeogenic and ureagenic enzymes in early-lactation dairy cows as measured by changes

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in the hepatic mRNA abundance of the ureagenic enzymes CPS-I, OTC, and ASS and of the gluconeogenic enzymes PC, PEPCK-C, and PEPCK-M.

MATERIALS AND METHODS

Experimental Design

The experiment was conducted at the Iowa State University Dairy Teaching Farm in Ames between May 2000 and April 2001. Cows were managed and treated in accordance with guidelines established by the Iowa State University Committee on Animal Care. Details about experimental design and sampling and analysis of milk, plasma, and liver, except for the analysis of hepatic gene expression, were described by Bobe et al. (2003a, b). Briefly, all cows were housed postpartum in a tie-stall barn and had free access to a diet formulated to meet NRC recommendations (NRC, 2001) and fed as a TMR. Fresh feed was offered daily at 0800 and 1800 h. Cows were milked daily at 0630 and 1830 h. Starting at 1400 h of d 8 postpartum, cows were injected s.c. every 8 h (0600, 1400, and 2200 h) for 14 d with 60 mL of 0.15 M NaCl solution (pH 10.25) that contained either 0 (control; $n = 3$) or 5 (glucagon; $n = 4$) mg of lyophilized bovine glucagon (donated by Eli Lilly & Co., Indianapolis, IN). The presented results are from cows at the end of the trial ($n = 7$) for which liver samples for mRNA analysis were taken and hepatic mRNA concentrations of gluconeogenic and ureagenic enzymes were determined. Except for one cow in the control group, all cows had 1 to 3% triacylglycerol of liver wet weight at d 8 postpartum.

Sample Collection and Chemical Analyses

Liver samples (1–3 g) were obtained by puncture biopsies at d -4 (range: -8 to -3), 3, 8, 11, 15, 22, 28, 36, and 43 postpartum. During the injection period, liver samples were collected 2 to 8 h after the morning injection. Statistical analysis for the mRNA concentrations was restricted to liver samples collected within 6 h of the last injection based on our prior observations on the duration of the glucagon effect on gene expression (She et al., 1999; Williams et al., 2006), which decreased the number of liver samples of glucagon-treated cows from 4 to 3 for d 11 and 15 postpartum. The time span between liver sampling and injection and feeding was similar between groups and among biopsy dates. Liver samples were blotted dry. One aliquot (1 g) was snap-frozen in liquid nitrogen and stored at -80°C until being analyzed for liver glycogen (Bobe et al., 2003b). Another aliquot (1 g) was placed in a tube containing 10 mL of guanidinium thiocyanate so-

lution [4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.4), 0.5% N-lauroyl sarcosine, and 0.1 M β -mercaptoethanol], snap-frozen in liquid nitrogen, and stored at -80°C until analysis. The gene expression analysis procedure, including cDNA probes, RNA extraction, and Northern analysis, has been described in detail previously (Velez and Donkin, 2005). The mRNA concentrations were adjusted by using a pooled sample of bovine liver RNA that was added within each run as external control and by using 18S rRNA concentrations as an internal control.

Blood samples were collected at 0700 h from the coccygeal vein or artery at d -4, 3, 8 (before first injection), 9, 11, 15, 22 (after last injection), 23, 28, 36, and 43 postpartum. Blood samples at d 9 and 23 postpartum were taken to measure short-term glucagon effects. During the injection period, blood samples were collected 1 h after the morning injection in 10-mL vacutainer tubes (Becton Dickinson and Co., Rutherford, NJ) containing $\text{K}_3\text{-EDTA}$, and plasma was prepared by centrifugation within 20 min. In addition, blood samples were collected on d 8 (after first injection), 15, and 21 postpartum at -15, -10, -5, 15, 30, 45, 60, 80, 100, 120, 150, 180, 210, 240, 300, 360, 420, and 480 min relative to the injection from 2 control and 3 glucagon-treated cows of this study (Bobe et al., 2003a). Plasma samples were stored at -20°C until analysis for glucose, insulin, plasma urea N, and α -amino N. Data for concentrations of plasma glucagon cannot be reported because, in contrast to $\text{Na}_3\text{-EDTA}$ and manufacturer's instructions, $\text{K}_3\text{-EDTA}$ interfered with the analysis of glucagon samples (glucagon kit number KGND1; Diagnostic Products Corp., Los Angeles, CA). Milk production was recorded for each milking until d 43 postpartum. Representative samples of the 2 milkings at d 3, 8 (before first injection), 11, 15, 22 (before last injection), 25, 28, 36, and 43 postpartum were analyzed separately for protein concentration by mid-infrared spectrophotometry (MilkoScan 203, Foss Food Technology, Eden Prairie, MN).

Statistical Analyses

Data were analyzed as a repeated measures study by using the mixed models procedure (PROC MIXED) of SAS version 9.1.3 (SAS Institute, 2004). The hepatic mRNA gene expression levels were normalized by $\log_2$ -transformation of PC, PEPCK-C, PEPCK-M, ASS, and CPS-I mRNA and by square-root transformation of OTC mRNA. The effects of glucagon injections on liver, plasma, and milk variables were evaluated by comparing the values during the injection period combined or each time point separately with that directly before the injection period at d 8 postpartum. Because of the

large variation in liver, plasma, and milk variables during the periparturient period that differs among cows, only the most immediate time point of d 8 postpartum was chosen as covariate. The fixed effects in the model were treatment (control, glucagon), time postpartum (time points differ depending on variable), and the interaction of treatment by time postpartum. A first-order autoregressive variance-covariance structure for time postpartum was used to account for repeated measures taken on individual cows across time. To obtain the correct degrees of freedom, the KENWARDROGER option was invoked. The KENWARDROGER option consists of the Satterthwaite adjustment for degrees of freedom with a Kenward-Roger adjustment on standard errors, which can be used for repeated measures studies. Significance was declared at $P \leq 0.05$, and trends toward significance were declared at $P \leq 0.10$. Means and standard errors of means (SEM) presented in figures are least squares means and the largest SEM of the time points.

RESULTS AND DISCUSSION

In ruminants, the primary effect of glucagon is to increase hepatic glucose synthesis (Brockman et al., 1975; Brockman, 1978). After liver glycogen stores are depleted, gluconeogenic, nonessential AA are a primary gluconeogenic precursor for hepatic glucose synthesis, especially during negative energy balance in early lactation (Hanigan et al., 2004). One of the mechanisms by which glucagon increases hepatic glucose synthesis is by increasing hepatic extraction of gluconeogenic, non-essential AA (Brockman et al., 1975; Brockman, 1978). To a lesser extent, glucagon increases hepatic lipolysis, fatty acid oxidation, and ketone synthesis (Brockman and Laarveld, 1986). Insulin, which is released by the pancreas in response to glucagon, then promotes the uptake of glucose and ketone bodies by extra-hepatic tissues (Brockman, 1978). We showed that multiple s.c. injections of glucagon over a 14-d period result in sustained increases in plasma glucose concentrations in early-lactation dairy cows (Bobe et al., 2003a; Nafikov et al., 2006; Osman et al., 2008). Previously, it was assumed that glucagon affects hepatic glucose synthesis only transiently (Brockman and Laarveld, 1986). One potential biological mechanism by which s.c. glucagon injections might increase hepatic glucose production is by changing concentrations of plasma glucagon and insulin, which, in turn, alter mRNA concentrations of ureagenic and gluconeogenic enzymes.

We reported that multiple s.c. injections of glucagon of 5 mg/d every 8 h for 14 d (same glucagon injection protocol as this study) consistently increased plasma glucagon concentrations in early-lactation dairy cows

for 3.5 to 5 h after injections (Osman et al., 2008). Although we were not able to measure plasma glucagon concentrations in this study, we assume that plasma glucagon concentrations were elevated in glucagon-treated cows at the time of liver biopsy because all analyzed liver biopsies were taken within 6 h (mean: 247 min; range: 160 to 345 min) after glucagon injection.

We reported that multiple s.c. injections of glucagon of 5 mg/d every 8 h for 14 d starting at d 8 postpartum increased plasma insulin concentrations in early-lactation dairy cows for at least 2 h at d 8 and 15 and for at least 3 h at d 21 postpartum (Bobe et al., 2003a). Similarly, we observed an increase in plasma insulin concentrations 1 h after glucagon injection in this study ($P = 0.03$; results not shown). By using the closest previous blood sampling time point to the liver biopsy, glucagon-treated cows had, compared with the last pre-glucagon sample (morning of d 8 postpartum), similar plasma insulin concentrations at d 8 (1.48 ± 1.65 mIU/L; $P = 0.37$), tended to have higher concentrations at d 15 (2.82 ± 1.62 mIU/L; $P = 0.09$), and had higher concentrations at d 21 postpartum (5.41 ± 1.42 mIU/L; $P = 0.0005$). Compared with saline-treated cows, glucagon-treated cows had similar plasma insulin concentrations at d 8 (4.34 ± 0.92 vs. 3.29 ± 1.30 mIU/L; $P = 0.52$) and 15 postpartum (5.30 ± 1.33 vs. 6.06 ± 1.30 mIU/L; $P = 0.68$) and higher concentrations at d 21 (7.88 ± 1.07 vs. 2.43 ± 1.30 mIU/L; $P = 0.003$). Thus, we assume that at the time of the liver biopsy, glucagon-treated cows had elevated plasma insulin concentrations at d 21 but not at d 11 or 15 postpartum.

During the injection period, we observed numeric increases in mRNA concentrations of key ureagenic enzymes (CPS-I: 2.3-fold; OTC: 1.5-fold; ASS: 1.8-fold) in liver samples from glucagon-treated cows, which was significant at d 11 postpartum (Figure 1, panels A to C). To our knowledge, our data are the first to report on the effect of glucagon on mRNA concentrations of ureagenic enzymes in dairy cows. Glucagon increases ureagenesis in bovine hepatocytes (Zhu et al., 2000). In rats, glucagon, or its secondary messenger cyclic AMP, increases mRNA concentrations (Ryall et al., 1986; Morris et al., 1987) and activities of CPS-I, OTC, and ASS (Snodgrass et al., 1978). Cell culture studies are inconclusive as to whether insulin might counteract the glucagon-induced increase of synthesis and activities of the urea cycle enzymes (Lin et al., 1982; Husson et al., 1987; Kitigawa, 1987). We conclude that transcription rates of ureagenic enzymes are responsive to hormonal regulation by glucagon in early-lactation dairy cows.

We hypothesize that an increase in transcription rates of ureagenic enzymes could mechanistically link the decrease in milk protein content and amount (Fig-

Figure 1. Effect of glucagon (5 mg injected every 8 h for 14 d) on hepatic mRNA expression of ureagenic enzymes, milk protein production, and concentrations of plasma α -amino N and plasma urea N ($n = 4$ glucagon; $n = 3$ saline). A) Effect of glucagon (overall: $P = 0.36$; d 11: $P = 0.05$; d 15: $P = 0.53$; d 22: $P = 0.81$) on carbamoylphosphate synthetase I (CPS-I). B) Effect of glucagon (overall: $P = 0.17$; d 11: $P = 0.04$; d 15: $P = 0.21$; d 22: $P = 0.64$) on ornithine transcarbamylase (OTC). C) Effect of glucagon (overall: $P = 0.12$; d 11: $P = 0.008$; d 15: $P = 0.37$; d 22: $P = 0.47$) on argininosuccinate synthetase (ASS). D) Effect of glucagon (overall: $P = 0.01$; d 11: $P = 0.01$; d 15: $P = 0.07$; d 22: $P = 0.03$) on milk protein production. E) Effect of glucagon (overall: $P = 0.53$; d 9: $P = 0.10$; d 11: $P = 0.87$; d 15: $P = 0.79$; d 22: $P = 0.78$) on α -amino N. F) Effect of glucagon (overall: $P = 0.72$; d 9: $P = 0.08$; d 11: $P = 0.79$; d 15: $P = 0.84$; d 22: $P = 0.86$) on plasma urea N. Hepatic mRNA concentrations of ureagenic enzymes did not differ ($P \geq 0.10$) between glucagon- and saline-treated cows at d 8 postpartum.

ure 1, panel D) and the tendency to decrease plasma α -amino N concentrations (20% decrease at d 9 postpartum; Figure 1, panel E; Bobe et al., 2003b) with the increase in plasma glucose concentrations during glucagon treatment (Figure 2, panel D; Bobe et al., 2003b). However, an increase in transcription rates cannot explain why plasma urea N concentrations were not consistently changed by multiple s.c. glucagon injections in this (Figure 1, panel E) and other studies (Nafikov et al., 2006; Osman et al., 2008). Probably, the difference between urea synthesis, recycling, and excretion is regulated differently than is urea synthesis. Despite the increase in plasma glucose concentrations, glucagon did not consistently change milk or milk lactose production (results not shown; Bobe et al., 2003b). One potential reason is that the mammary gland lacks glucagon receptors because glucagon does not bind to or affect synthesis rates in extracted mammary tissue (Wilde and Kuhn, 1981; Jones et al., 1984; Robson et al., 1984). In addition, the glucose concentrations in Figure 2, panel D were measured at the maximal response to glucagon 1 h after the injection and, thus, plasma glucose concentrations fluctuate between glucagon injections and the average plasma glucose increase is smaller than 1 h after the injection (Bobe et al., 2003a).

During the injection period, we observed increases in mRNA concentrations of key gluconeogenic enzymes (PC: 7.1-fold; PEPCK-M: 4.4-fold; PEPCK-C: 3.7-fold) in glucagon-treated cows (Figure 2, panels A to C). Similarly, Williams et al. (2006) reported an increase in mRNA concentrations for PC, PEPCK-M, and PEPCK-C (1.5-fold) in mid-lactation cows receiving s.c. glucagon. The smaller magnitude of their observed changes compared with those of this study might be explained by the lower glucagon dosage used (3.75 vs. 5 mg). Other potential contributing factors are that cows had lower baseline glucagon and glucose concentrations, higher baseline insulin concentrations, and were in a positive energy balance. She et al. (1999) reported an increase in mRNA concentrations of PC (1.5-fold) but a decrease in mRNA concentrations of PEPCK-C (3.5) in mid-lactation cows receiving i.v. glucagon at 0.625 mg/h for 3.5 h. We conclude that transcription rates of gluconeogenic enzymes are responsive to hormonal regulation by glucagon in early-lactation dairy cows.

Glucagon increased mRNA concentrations of PC (7.1-fold) to a greater degree than those of PEPCK-M (4.4-fold) and PEPCK-C (3.7-fold; Figure 1, panels A to C). In agreement with our results, Brockman and Manns (1974) reported that 3- and 24-h infusions of glucagon increased enzyme activities of PC but not PEPCK in ovine liver. In addition, She et al. (1999) reported that infusions of glucagon increase mRNA concentrations of PC but not of PEPCK-C in mid-lactation dairy cows. In

rat hepatocytes, glucagon increases transcription rates of PEPCK-C (Beale et al., 1984; Christ et al., 1988), whereas insulin counteracts the effect of glucagon on transcription rates and accelerates mRNA PEPCK-C degradation (Christ et al., 1988, 1990; Christ and Nath, 1993). It is not well understood how hormones regulate the transcription rates of PC and PEPCK-M (Jitrapakdee and Wallace, 1999; Beale et al., 2007). We theorized that PC and PEPCK are regulated by hormones in parallel to maintain gluconeogenic flux. However, the hepatic demand for mitochondrial oxaloacetate includes carbon flux for gluconeogenesis and synthesis of the tricarboxylic acid cycle intermediates for oxidation of acetyl CoA that originated from β -oxidation of fatty acids and from ketogenic AA catabolism. Thus, we cannot exclude the possibility that hormones regulate the transcriptional and degradation rates of PC and PEPCK differently in response to metabolic demand and substrate supply.

With the progression of the glucagon injection period, the increases in mRNA concentrations of gluconeogenic and ureagenic enzymes were attenuated (PC: 13.0- to 1.6-fold; PEPCK-M: 7.8- to 1.9-fold; PEPCK-C: 6.3- to 1.2-fold; CPS-I: 2.8- to 0.9-fold; OTC: 1.7- to -1.1-fold; ASS: 2.0- to 1.3-fold; Figure 1, panels A to C; Figure 2, panels A to C). The stronger glucagon-induced changes in mRNA concentrations of gluconeogenic versus ureagenic enzymes are consistent with the primary role of glucagon to increase hepatic glucose synthesis (Brockman et al., 1975; Brockman, 1978). Consistent with our findings, She et al. (1999) observed a decrease in glucagon response of mRNA concentrations of PEPCK-C when early-lactation cows were infused i.v. for 14 d with 10 mg/d of glucagon. Potential explanations for the attenuated glucagon responses are an increase in insulin concentrations, a feedback loop sensing the improved carbohydrate status as indicated by increased plasma glucose and liver glycogen concentrations (Brockman and Laarveld, 1986; Cherrington et al., 1987; She et al., 1999), or a decrease in the number of hepatic glucagon receptors (Authier and Desbuquois, 2008). Plasma insulin concentration was unchanged at liver biopsy time only in the first injection week. Liver glycogen concentration decreased in the first injection week and then increased (Figure 2, panel E; Bobe et al., 2003b). Thus, transcriptional rates of gluconeogenic and ureagenic enzymes might be responsive to glucagon, insulin, and the carbohydrate status of the cow.

During the glucagon injection period, responses of plasma glucose (Figure 2, panel D) and mRNA concentrations of gluconeogenic and ureagenic enzymes (Figure 1, panels A to C; Figure 2, panels A to C) were not parallel, which is consistent with observations by She et al. (1999). Explanations for the observed dif-

Figure 2. Effect of glucagon (5 mg injected every 8 h for 14 d) on hepatic mRNA expression of gluconeogenic enzymes and concentrations of plasma glucose and liver glycogen ($n = 4$ glucagon; $n = 3$ saline). A) Effect of glucagon (overall: $P = 0.02$; d 11: $P = 0.001$; d 15: $P = 0.03$; d 22: $P = 0.57$) on pyruvate carboxylase (PC). B) Effect of glucagon (overall: $P = 0.003$; d 11: $P < 0.0001$; d 15: $P = 0.03$; d 22: $P = 0.14$) on mitochondrial phosphoenolpyruvate carboxykinase (PEPCK-M). C) Effect of glucagon (overall: $P = 0.04$; d 11: $P = 0.007$; d 15: $P = 0.08$; d 22: $P = 0.45$) on cytosolic phosphoenolpyruvate carboxykinase (PEPCK-C). D) Effect of glucagon (overall: $P = 0.001$; d 9: $P = 0.02$; d 11: $P = 0.01$; d 15: $P = 0.01$; d 22: $P = 0.002$) on plasma glucose. E) Effect of glucagon (overall: $P = 0.38$; d 11: $P = 0.21$; d 15: $P = 0.29$; d 22: $P = 0.43$) on liver glycogen. Hepatic mRNA concentrations of gluconeogenic enzymes did not differ ($P \geq 0.10$) between glucagon- and saline-treated cows at d 8 postpartum.

ferences in glucagon response are that glucagon and insulin regulate plasma glucose concentrations through multiple mechanisms. Besides changing transcription rates of gluconeogenic and ureagenic enzymes, glucagon and insulin can influence the flux of substrates and products of gluconeogenesis by altering a) the supply of substrates such as the uptake of amino acids from the circulation (Brockman, 1978); b) the phosphorylation state of key regulatory hepatic enzymes such as pyruvate kinase (Pilkis et al., 1988); c) enzyme activities by changing the availability of activators such as acetyl CoA for PC (She et al., 1999); and d) mRNA degradation rates of gluconeogenic enzymes such as PEPCK-C (Christ et al., 1988, 1990).

In addition, mRNA and protein concentrations of gluconeogenic and ureagenic enzymes are not parallel. For example, the half-lives of ureagenic enzymes are 3 to 9 d compared with only a few hours for their mRNAs (Morris, 1992). Maximal mRNA concentrations of PEPCK-C, which were observed 2 h after glucagon administration, preceded maximal enzyme activities by 2 h (Christ et al., 1988, 1990). Thus, choosing the appropriate biopsy time is critical for observing a glucagon response. Furthermore, acute and prior exposure to glucagon and insulin both might influence mRNA concentrations of ureagenic and gluconeogenic enzymes at the time of liver biopsy.

In conclusion, our results suggest that glucagon orchestrates gene expression of gluconeogenic and ureagenic enzymes to increase hepatic glucose synthesis in early-lactation dairy cows. A coordinated regulation of ureagenesis and gluconeogenesis is important because ureagenesis and gluconeogenesis compete for cytosolic oxaloacetate as substrate. In addition, the gluconeogenic precursor malate is synthesized in the urea cycle from argininosuccinate and the urea cycle detoxifies ammonia, which is a deamination product of gluconeogenic AA. Besides glucagon, insulin and carbohydrate status of the dairy cow might play a role in the regulation of transcription rates of ureagenic and gluconeogenic enzymes. Future larger studies should verify our results.

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