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^{13}C NMR Study of Hepatic Pyruvate Carboxylase Activity in Tumor Rats

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Summary. Alanine and lactate, as major gluconeogenic substrates, must be converted into oxaloacetate by way of pyruvate carboxylase before their entry into gluconeogenesis. Although it is well known that hepatic gluconeogenesis from these substrates is increased in tumor hosts, the involvement of pyruvate carboxylase has not been demonstrated. In the present study, we examined pyruvate carboxylase activity in the perfused livers of tumor rats using ^{13}C NMR spectroscopy with [3-^{13}C]-alanine as the gluconeogenic substrate. A substantial increase in hepatic [3-^{13}C]-aspartate production was found in the tumor rats. Since aspartate accumulation directly reflects fluxes of alanine through pyruvate carboxylase, the observed increase in hepatic production of [3-^{13}C]-aspartate in tumor rats indicates that pyruvate carboxylase activity is significantly enhanced.

Abnormal gluconeogenic substrate utilization in the livers of tumor-bearing hosts has been demonstrated by many studies [1, 2]. In order to better understand and possibly better control such abnormalities, it is essential to delineate the specific alterations in hepatic enzymatic pathways involved. Pyruvate carboxylase plays an important role in determining the rate of hepatic glucose production for most gluconeogenic substrates including alanine and lactate, with the exception of glycerol. Therefore, pyruvate carboxylase activity provides important information regarding the utilization of gluconeogenic substrates by the liver. In the present study, we examined the pyruvate carboxylase activity in perfused livers of tumor-bearing rats using ^{13}C NMR spectroscopy. This was accomplished by monitoring the amount of [3-^{13}C]-aspartate appearing in the liver and in the perfusion medium when [3-^{13}C]-alanine was used as the substrate.

Materials and Methods

Animals The experimental setup was modified from Cohen [3] and described previously [4]. Briefly, Fischer 344 female rats weighing between 125 to 135 gm were inoculated with R3230AC mammary adenocarcinoma [5, 6] (N=4). Weight matched normal rats were pair-fed and were used as controls (N=4).

Liver perfusion Five weeks after tumor inoculation (or pair-feeding), the rats were fasted for 24 hours and anesthetized with ether. The livers were removed and perfused with a

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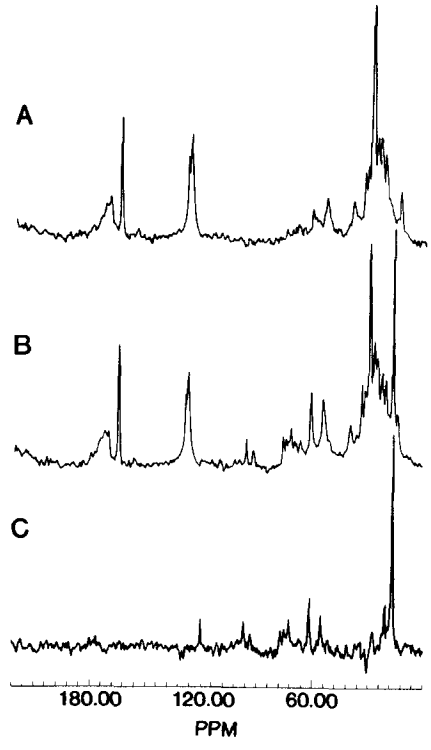

Figure 1. Typical ^{13}C NMR background spectrum (A) of a normal liver, spectrum (B) for the same liver perfused with L-[3-^{13}C]-alanine, and spectrum (C) was obtained by subtracting spectrum (A) from spectrum (B).

Krebs bicarbonate buffer containing 2% bovine albumin and 5 mM glucose. During its passage through the oxygenator, the perfusion medium was also warmed to 37° C. The final pH of the perfusion medium was maintained between 7.35 - 7.45 and the medium was recirculated at 5-6 ml/gm-liver/min.

NMR Spectroscopy A Nicolet 200 MHz spectrometer with a Helmholtz coil and a Waltz unit was employed. The recycling time was 0.78 seconds and the pulse angle was 45°. For each spectrum, 2308 acquisitions were obtained over a 30-minute period. To obtain a ^{13}C natural abundance background spectrum, the liver was perfused by a medium without L-[3-^{13}C]-alanine initially (Figure 1A). Then, the medium was replaced by one to which 8 mM L-[3-^{13}C]-alanine had been added, and three more spectra were acquired (Figure 1B).

Data Analysis The NMR data were processed and analyzed using a VAX 11/750 minicomputer. Prior to data analysis, the background spectrum was subtracted from the [3-^{13}C]-alanine perfused liver spectra (Figure 1C). The [3-^{13}C]-aspartate peak heights in the subtraction spectra of the perfused livers and the perfusion medium spectra were compared between the tumor and pair-fed rats using the Analysis of Variants (ANOVA) test.

Results

The [3-^{13}C]-aspartate peak, appearing at 37 ppm [3], is discrete and clearly defined (Figure 2). The experimental conditions of the NMR spectrometer were sufficiently constant that no appreciable changes in the line widths and the ppm positions of the [3-^{13}C]-aspartate peak were observed. Thus, the absolute peak heights were used in the comparison between the tumor and pair-fed rats (Table 1). We found significantly greater

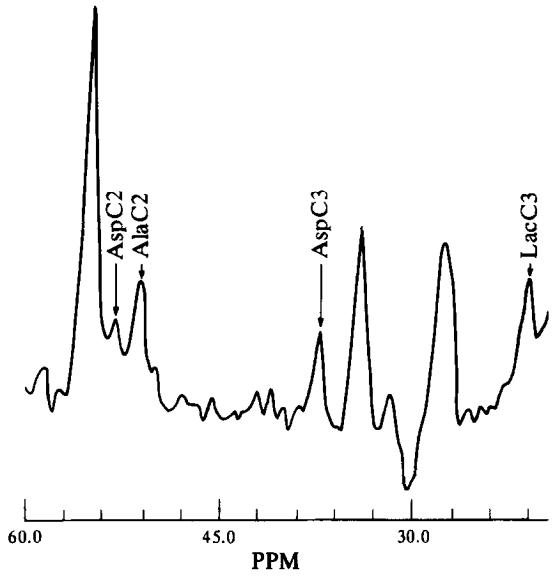

Figure 2. A typical ^{13}C NMR subtraction spectrum for the perfused liver of a normal rat. Note the [3-^{13}C]-aspartate peak at 37 ppm as indicated by "AspC3".

peak heights of [3-^{13}C]-aspartate in the livers of tumor rats than in the pair-fed ones ($p < 0.01$).

Discussion

Many methods have been developed to measure the activities of pyruvate carboxylase in the liver directly [7, 8, 9, 10, 11]. With these methods, the liver is freeze-clamped and the mitochondria isolated prior to the determination of pyruvate carboxylase activities. Thus, the pyruvate carboxylase activities are measured in nonviable tissues. In the present study, the pyruvate carboxylase activity was assessed through the accumulation of [3-^{13}C]-aspartate in perfused livers using ^{13}C NMR spectroscopy. Compared with other methods, NMR spectroscopy has the unique advantage of providing information regarding a viable liver under near physiological conditions.

Table 1. The means and standard deviations for the peak heights of the [3-^{13}C]-aspartate in the livers of pair-fed and tumor rats ($p < 0.01$)

	Time	Pair-fed	Tumor
AspC3	0-30 min	0.10 ± 0.12	1.28 ± 0.49
	30-60 min	0.05 ± 0.09	1.28 ± 0.62
	60-90 min	0.03 ± 0.05	1.12 ± 0.74

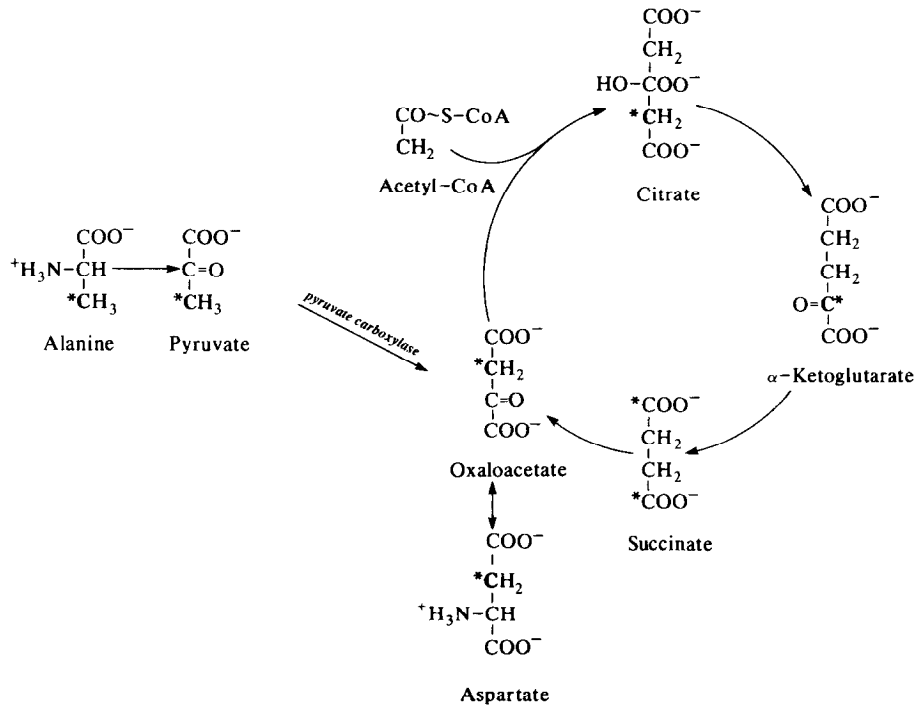

Figure 3. The labeling of oxaloacetate and aspartate through the pyruvate carboxylase pathway. * indicates the carbons labeled with ^{13}C.

[3-^{13}C]-alanine was selected as the substrate because it is the principal amino acid utilized by the liver for gluconeogenesis under normal conditions [12]. After entering the liver, [3-^{13}C]-alanine is converted into [3-^{13}C]-pyruvate through transamination (Figure 3). Then, [3-^{13}C]-pyruvate enters the gluconeogenic pathway after it is metabolized into [3-^{13}C]-oxaloacetate by pyruvate carboxylase. Thus, the production of [3-^{13}C]-oxaloacetate reflects the pyruvate carboxylase activity. Meanwhile, oxaloacetate is involved in other pathways. It is also generated from pyruvate through pyruvate dehydrogenase and the Krebs cycle, and consumed by the gluconeogenic and transamination processes.

Although both pyruvate carboxylase and pyruvate dehydrogenase are involved in the generation of oxaloacetate, in fasted animals as in the present study, much smaller quantities of pyruvate are decarboxylated than carboxylated [13, 14, 15]. Therefore, under this condition, pyruvate carboxylase dominates the production of [3-^{13}C]-oxaloacetate from [3-^{13}C]-pyruvate. Existing evidence also suggests that equilibration takes place between oxaloacetate and fumarate [13, 16, 17, 18]. During this process, the ^{13}C labeling at oxaloacetate C3 is redistributed equally between C2 and C3. Should the rate of oxaloacetate metabolism exceed its rate of equilibration, only partial equilibration would occur. Hence, the equilibration process dilutes the labeling at oxaloacetate C3.

As a result of being the crossing point for all these metabolic processes, oxaloacetate does not accumulate significantly. On the other hand, through

transamination, oxaloacetate directly gives rise to aspartate which does accumulate. Thus, aspartate may serve as a direct indicator for oxaloacetate metabolism.

Therefore, the increased [3-^{13}C]-aspartate found in the livers of tumor rats suggests a more rapid production of [3-^{13}C]-oxaloacetate compared to the pair-fed rats. This augmented oxaloacetate production is most likely due to an enhanced flux through pyruvate carboxylase, in view of the above considerations. In addition, a rise in the rate of oxaloacetate transamination may contribute to the increased aspartate generation. A reduced consumption of oxaloacetate through gluconeogenesis may also play a part in the livers of tumor rats; however, this is highly unlikely. As observed in previous studies [4, 19, 20, 21, 22, 23, 24], increased, rather than decreased, hepatic gluconeogenesis is found in tumor-bearing hosts.

In summary, our study has utilized the accumulation of a direct metabolite of oxaloacetate, namely, aspartate, to evaluate its rate of production, and has found greatly enhanced pyruvate carboxylase activity in the presence of distant tumor. We believe this increased activity may be partly responsible for the augmented gluconeogenesis frequently observed in tumor hosts. Further studies are required to determine whether changes in the total quantity, and/or in the reaction rate, of pyruvate carboxylase are responsible for this finding.

Acknowledgments

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